II. REMARKS

A. Status of the Claims

Claims 1-4, 6, 7, 11 and 12 are currently pending. Claims 5, 8-10 and 13-20 have been cancelled without prejudice. Claims 1-3, 6, 11 and 12 have been amended without prejudice to replace the phrase "non-human animal" with the term "mouse". Support for this amendment can be found throughout the application as originally filed, e.g., in the Examples. It is respectfully submitted that no new matter has been added by virtue of this amendment.

B. Priority

In the Office Action, the Examiner alleged that the instant application was not filed before the abandonment of parent application serial no. 09/763,117, abandoned on April 15, 2003.

In response, Applicants submit herewith, as Exhibit A, copies of the Petition for Revival of an Application for Patent Abandoned Unintentionally Under 37 CFR 1.137(b) and a Petition for Three Month Extension of Time (extending the period of time for response from April 14, 2003 to July 14, 2003), filed October 19, 2005 in parent application U.S. Serial No. 09/763,117.

The Examiner is respectfully requested that the priority claim of the instant application be acknowledged upon the granting of the above-referenced petitions.

C. Rejections

Initially, Applicants wish to thank the Examiner for removing the previous objections and rejections over the drawings and of the claims under 35 U.S.C. §§ 101, 112, first and second paragraphs and 102(b).

1. Claim rejections under 35 U.S.C. §112

In the Office Action, the Examiner rejected claims 1-4 and 6-20 under 35 U.S.C. §112, first paragraph. The Examiner alleged that the specification does not provide enablement for one of skill in the art "to make and use the invention commensurate in scope with these claims", and

specifically referred to the terms "transgenic non-human animal" and "neuron specific promoter" as being non-enabled.

In response to the rejection of the term "transgenic non-human animal", Applicants have amended the present claims to recite a "transgenic mouse". With respect to the Examiner's position that "the art teaches that the isolation of promoters is a lengthy, unpredictable process", Applicants respectfully disagree. The prior art has shown that there are numerous promoters specific to particular neurons. As evidence of such, Applicants submit the following table, with the references cited attached herewith as Exhibit B:

No.	Name of Promoter	Neuron	Reference
1	Tyrosine hydroxylase gene promoter	Catecholaminergic neurons	Mol. Brain Res. 16:274-286 (1992)
2	Dopamine beta-hydroxylase gene promoter	Noradrenergic neurons	Mol. Brain Res. 17:239-244 (1993)
3	Choline acetyltransferase gene promoter	Cholinergic neurons	Porc. Natl. Acad. Sci. USA 92:4046-4050 (1995)
4	Insulin gene promoter	Medial habenula cholinergic neurons	Biol. Cell. 85: 137-146 (1995)
5	Gonadotropin-releasing hormone gene promoter	Gonadotropin-releasing hormone neurons	J. Biol. Chem. 271: 20018-20023 (1996)
6	GABA-A receptor alpha6 subunit gene promoter	Cerebellar granule cells	J. Neurochem. 67: 907-916 (1996)
7	Serotonin transporter gene promoter	Raphe nucleus serotonergic neurons	J. Neurochem. 70: 932-939 (1998)[1]

In response to the Examiner's assertion that "neurons that are affected in [ALS] are motor neurons [and]...[t]he specification, at the time of filing, does not teach how to make or use a promoter that expresses specifically in motor neurons", Applicants respectfully direct the Examiner's attention to the choline acetyltransferase gene promoter, listed as No. 3 in the Table above. As choline acetyltransferase has the ability to express transgene in motor neurons as well, Applicants submit that the neuron-specific promoter recited in the present claims is also applicable for the neurons that are affected in ALS.

[1] It is noted that this reference was published the same year, i.e., 1998, as the priority date of the instant application. It is further noted that the month of publication of the reference is unkown.

In view of the amendments made and the arguments presented, Applicants respectfully request that the rejection under 35 U.S.C. §112 be removed.

2. Claim rejections under 35 U.S.C. §102

In the Office Action, the Examiner rejected claims 1-4 and 6-20 under 35 U.S.C. § 102(b) as being anticipated by PCT/JP99/04439, filed August 18, 1999. The Examiner indicated that "this rejection may be withdrawn if the Applicant provides a translation of the PCT and if the Applicant establishes the continuity of this application."

In response, Applicants submit herewith, as Exhibit C, an English translation of International Application PCT/JP99/04439, which the instant application claims priority to. Applicants additionally note that continuity of the parent application to the instant application has been addressed above, in subsection B of this communication. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. §102(b) over PCT/JP99/04439 be removed.

In the Office Action, the Examiner also rejected claims 1-3 and 8-12 under 35 U.S.C. § 102(b) as being anticipated by Yoshihara et al. (1999, Neuron, 22: 33-41).

In response, Applicants respectfully point out that the instant application claims priority to Japanese Patent Application No. 232817/1998, filed April 19, 1998. Therefore, Applicants submit that the Yoshihara et al. is not prior art, as the publication date of the reference, i.e., January 1999, occurs after the date of priority of instant application, i.e., April 19, 1998.

Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 102(b) over Yoshihara et al. be removed.

3. Claims rejection under 35 U.S.C. §103

In the Office Action, the Examiner rejected claims 4, 6, 7 and 13-20 under 35 U.S.C. 103(a) as being unpatentable over Yoshihara et al. (1999, Neuron, 22: 33-41).

This rejection is traversed. Applicants respectfully submit, for the reasons stated above, that Yoshihara et al. is not prior art as applied to the instant application. Therefore, Applicants request that the rejection under 35 U.S.C. §103(a) be removed.

III. CONCLUSION

It is now believed that the above-referenced rejections have been obviated and it is respectfully requested that the rejections be withdrawn. It is believed that all claims are now in condition for allowance.

An early and favorable action on the merits is earnestly solicited. The Examiner is invited to contact the undersigned at the telephone number provided below if he believes that a telephonic interview will advance the prosecution of this application.

Respectfully submitted,
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EXHIBIT A

EXHIBIT B

EXHIBIT A

Molecular Brain Research, 16 (1992) 274–286

BRESM 70523

Analysis of the human tyrosine hydroxylase promoter–chloramphenicol acetyltransferase chimeric gene expression in transgenic mice

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(Accepted 21 July 1992)

Key words: Tyrosine hydroxylase; Promoter activity; Chloramphenicol acetyltransferase; Transgenic mouse; Catecholaminergic neuron; Immunocytochemistry

To investigate *cis*-elements responsible for catecholaminergic (CAnergic) neuron-specific expression of the tyrosine hydroxylase (TH) gene, we produced lines of transgenic mice carrying 5.0-kb, 2.5-kb and 0.2-kb fragments from the 5'-flanking region of the human TH gene fused to a reporter gene, chloramphenicol acetyltransferase (CAT), and designated them as TC 50, TC 25, and TC 02, respectively, and reporter gene expression in transgenic mice was analyzed by CAT assay by immunocytochemistry with anti-CAT antibody. High-level CAT expression was observed in the brain and adrenal gland using the 5.0-kb promoter of the TC 50 mice, but ectopic expression was consistently observed in several somatic tissues, e.g. thymus, colon, and testis. In brain, expression was achieved in CAnergic neurons with the largest construct (5.0 kb), but not with 2.5 kb or 0.2 kb of 5' flanking sequence. However, TC 50 mice also expressed CAT immunoreactivity in non-CAnergic neurons. In the TC 25 line CAT immunoreactivity was detected only in some non-CAnergic neurons. In the TC 02 line no CAT immunoreactivity was detected in any of the tissues examined. These results indicate that the 5.0-kb DNA fragment of the TH gene upstream region contains activity to express CAT in CAnergic neurons and surprisingly, lacks some regulatory elements attenuating ectopic expression, and that the 2.5-kb and 0.2-kb fragment are not sufficient for the proper expression. We discuss the presence of the tissue-specific regulatory elements in the structure portion of the TH gene and/or 3'-flanking region.

INTRODUCTION

Tyrosine hydroxylase (TH; tyrosine 3-monooxygenase; L-tyrosine, tetrahydropteridine: oxygen oxidoreductase; EC 1.14.16.2.) is the first and rate-limiting enzyme in catecholamine (CA) biosynthesis[18,28]. The enzyme is localized in catecholaminergic (CAnergic) neurons of central and peripheral nervous systems and in chromaffin cells of the adrenal medulla. CAnergic neurons are classified into three subtypes: dopamine (DA), norepinephrine (NE), and epinephrine (EPI) neurons; and chromaffin cells, into two types; NE- and EPI-producing cells. Differences between these cells are based on the presence of four CA-synthesizing enzymes. DA neurons contain TH and aromatic L-amino acid decarboxylase (AADC). NE-producing cells contain dopamine-β-hydroxylase (DBH) in addition to TH and AADC. EPI-producing cells have these three enzymes, as well as phenylethanolamine *N*-methyltransferase (PNMT). The presence of these CA-synthesizing enzymes in each CAnergic neuron has been well investigated by neuroanatomical analyses[10].

To study the regulation of CAnergic neuron-specific gene expression, we have focused our attention on the regulation of TH gene expression. Since TH is present in all three types of CA-producing cells, there is a possibility that the regulatory elements contributing to TH expression are common to those for the AADC, DBH, and PNMT genes. Many factors such as cyclic AMP (cAMP), glucocorticoids, nerve growth factor,

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and epidermal growth factor have been reported to modulate TH gene expression[5,19-21,35]. Recently, the human TH gene was isolated and characterized[14,17,29]. In the 5'-flanking region of the human TH gene, several sequences homologous to some regulatory elements, such as GC-box, AP-1 binding site, and cAMP response element, were identified, and found to be conserved in other species, i.e. mouse[11], rat[8], and bovine[3]. Since the expression pattern of TH is spatially and temporally specific, we have been utilizing transgenic mice to define the regulatory elements for gene expression. In a previous paper, we reported the production and analysis of transgenic mice carrying an 11-kb fragment containing the entire human TH gene[13]. The transgene was expressed strongly and tissue-specifically in the brain and adrenal gland. The results indicated that the transgene should encompass *cis*-elements driving CAnergic neuron-specific gene expression.

In the present work, to further clarify regulatory elements for tissue-specific gene expression, we analyzed the promoter activity of the 5'-flanking region of the TH gene. We generated several transgenic mouse lines carrying three serial fragments of the human TH 5'-flanking region fused to a reporter gene, chloramphenicol acetyltransferase (CAT). Analysis of gene expression was performed by measurement of CAT activity and by immunocytochemical analysis with newly produced anti-CAT antibody.

MATERIALS AND METHODS

Construction of transgenes

The plasmid pBstN-CAT derived from pKCRH2[24] contains the CAT gene, the second intron and the polyadenylation signal of the rabbit β-globin gene, and the polyadenylation signal of the SV40 early gene situated between the *Bam*HI and *Xho*I sites of Bluescript vector. The 5'-flanking region from *Xba*I site (~ 5 kb) to nucleotide position +10 was derived from the human TH genomic clone gHTH-E20[14], and the 3'-end of the fragment was modified to a *Sal*I site. The *Xba*I-*Sal*I (modified as blunt ended) fragment was introduced between the *Xba*I and *Bam*HI (modified as blunt ended) sites of pBstN-CAT to generate plasmid pTC. The 7.5-kb linear DNA fragment for the TC 50 gene, the 5.0-kb fragment for the TC 25 gene, and the 2.7-kb fragment for the TC 02 gene were produced by *Xba*I/*Xho*I, *Hin*dIII/*Xho*I, and *Sac*II/*Xho*I double digestion of plasmid pTC, respectively. These DNA fragments were separated by 0.7% agarose gel electrophoresis using Tris-acetate buffer[22].

Production of transgenic mice

DNA was purified from the agarose gel by repeated phenol extraction followed by ethanol precipitation. The precipitate was dissolved in Dulbecco's phosphate-buffered saline (PBS) at a concentration of about 10 μg/ml. After centrifugation, the supernatant was microinjected (~ 2 pl) into male pronuclei of fertilized C57BL/6J × DBA/2J F2 mouse eggs according to the method described by Gordon et al.[6]. Microinjected eggs were incubated overnight at 37°C, 5% CO_2 in M16 media and allowed to develop to the two-cell stage. Surviving two-cell stage eggs were transplanted into the oviducts of pseudopregnant foster mothers.

Integration of the transgene into the mouse genome was assessed by Southern blot analysis of DNA obtained from tail segments of all mice delivered. Tail DNA was digested with *Eco*RI alone or both *Eco*RI and *Bam*HI, electrophoresed on a 0.7% agarose gel, and transferred to a nylon membrane (Micron Separation Inc.). The TC 50 gene or the 1.2-kb *Acc*I-*Sal*I DNA fragment of the plasmid pTC was labeled by use of a multiprime labeling kit (Amersham) and [α-^{32}P]dCTP (Amersham). Hybridization was performed in a solution of 50% (v/v) formamide, 5×SSPE, 5×Denhardt's solution, 0.3% SDS, sonicated and heat-denatured salmon sperm DNA (250 μg/ml), and the probe for 16–20 h at 42°C. The membrane was washed successively in 2×SSC, 0.1% SDS for 30 min at 42°C, and in 0.1×SSC, 0.1% SDS for 20 min at 65°C. Transgene copy number was estimated by comparison of the intensity of the transgene with that of a series of known amounts of standard DNA.

CAT assay

Adult mice between 3 and 5 months of age were sacrificed by cervical dislocation. After dissection, tissues were homogenized in 0.25 M Tris-HCl (pH 7.5) with a sonicator. Homogenates were heated at 65°C for 15 min to inactivate inhibitory factors, centrifuged, and the supernatants were stored frozen until assayed. The protein concentration of each homogenate was determined with a Bio-Rad protein assay kit[2]. The analysis of CAT activity was performed as described previously[7,30]. Each assay contained 50 μg of tissue protein.

Production of anti-CAT antiserum

The CAT-expression vector was constructed as follows; The 0.7-kb *Bam*HI–*Bam*HI fragment containing the *E. coli* CAT protein-coding sequences was excised from pCM4 (Pharmacia), blunt-ended with Klenow fragment, and inserted into the *Sma*I site of a prokaryotic expression vector, pKK223-3 (Pharmacia). CAT protein was produced in *E. coli* JM109 transformed with the CAT-expression vector and purified as described previously[33]. A polyclonal antiserum was raised by immunization of rabbits with purified CAT protein in Freund's adjuvant[25,26].

Immunoblot analysis

The extracts from bacterial cells transformed with CAT-expression vector and non-transformed cells were subjected to 10% SDS–PAGE[16]. Protein bands were electrophoretically transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad Laboratories), and CAT protein was detected by incubation with anti-CAT antiserum, followed by incubation with ^{125}I-labeled protein A as described previously[13]. Non-transformed bacterial cells instead of transformed cells or preimmune rabbit serum instead of anti-CAT antiserum were used for negative control experiments.

Tissue processing

Adult mice (3 ~ 5 months old) were anesthetized with Nembutal (50 mg/kg, i.p.) and perfused intracardially with saline followed by 5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 6 min. Fixed tissues were dissected out and immersed overnight in the same fixative at 4°C. After rinsing with 10–30% sucrose in phosphate buffer for 2 days, cryostat sections (40 μm thick) through the frontal plane of the whole brain were collected in 0.1 M phosphate buffer (pH 7.4) for immunocytochemistry as described below. The adrenal glands from male mice were also processed as described above.

Immunocytochemistry

Immunocytochemistry was performed by the peroxidase–antiperoxidase (PAP) method[34] as described previously[25,26]. Sections (40 μm thick) were treated with 0.5% hydrogen peroxide for 10 min and washed with PBS. They were blocked with 5% normal swine serum for 30 min, washed, and incubated for 2 days at 4°C in rabbit anti-TH antiserum diluted to 1:4,000–10,000 or anti-CAT antiserum diluted to 1:3,000 in 0.01 M phosphate buffer containing 0.05% sodium azide. After a wash with PBS, the sections were incubated for 1 h at room temperature in swine anti-rabbit immunoglobulins (Dakopatts,

Z196) at a dilution of 1:100, washed, and then incubated with the PAP complex (Dakopatts, Z113) at a dilution of 1:200 for an additional hour. The complex was visualized with 0.4 mg/ml of 3,3'-diaminobenzidine tetrahydrochloride in the presence of 0.01% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.6). Sections were mounted on slides, dried, dehydrated, and then protected with coverslips. For electron microscopy, tissue sections were osmicated, embedded in epon, and ultrathin sectioned.

RESULTS

Production of transgenic mice

The nucleotide sequences from 5.0 kb, 2.5 kb, and 0.2 kb upstream of the transcription initiation site to nucleotide at position +10 of the human TH gene[14] were fused to the bacterial CAT protein-coding region[7] to generate chimeric genes designated TC 50, TC 25, and TC 02, respectively (Fig. 1). The fused genes were microinjected into fertilized C57BL/6J × DBA/2J F2 (B6D2F2) mouse eggs. Transgenic mice were identified by hybridization of mouse tail DNA with the TC 50 gene used as a probe. Since there is one *Eco*RI recognition site in the transgene, the *Eco*RI restriction enzyme was used in Southern blot analysis. Hybridization patterns from head-to-tail concatemers of intact integrated TC genes yielded distinctively characteristic bands of 7.5, 5.0, and 2.7 kb for the TC 50, TC 25, and TC 02 genes, respectively. Eleven independent founders carrying the TC 50 gene, eight founders carrying the TC 25 gene, and eleven founders carrying the TC 02 gene were generated. All weaned founders were mated with B6D2F1 mice, and Southern hybridization of tail DNA was carried out from offspring of independent founders. As shown in Table 1, 6 out of 11 TC 50 founder mice, 7 out of 8 TC 25 founder mice, and 9 out of 11 TC 02 founder mice stably transmitted the transgenes to their offspring. Inheritance of the transgene was autosomal and followed Mendelian inheritance. Every transgenic littermate in all lines analyzed showed the same Southern hybridization pattern and intensity as that of the corresponding founder mouse. This strongly suggests the transgene insertion at a single site without any DNA rearrangement or any segregation in each transgenic line. The copy number of the transgenes varied from 1 to 50 per cell among transgenic lines.

CAT expression in the TC 50, TC 25 and TC 02 transgenic mice

To examine the promoter activity in the brain of the three transgene constructs, TC 50, TC 25, and TC 02, we assayed CAT activity in the brains from independent transgenic lines. The brain tissues were obtained from offspring of all the transgenic lines and from the founders that were unable to transmit the transgene to offspring. CAT activity was measured in whole-brain extracts. As summarized in Table 1, all TC 50 mice examined, 2 out of 6 TC 25 mice, and 6 out of 9 TC 02 mice had CAT activity in their brains. Non-transgenic littermates were examined as negative control animals. Since no CAT activity was observed in any non-transgenic tissues (data not shown), the presence of CAT activity in transgenic tissues directly reflects transgene expression. To investigate the tissue-specific expression

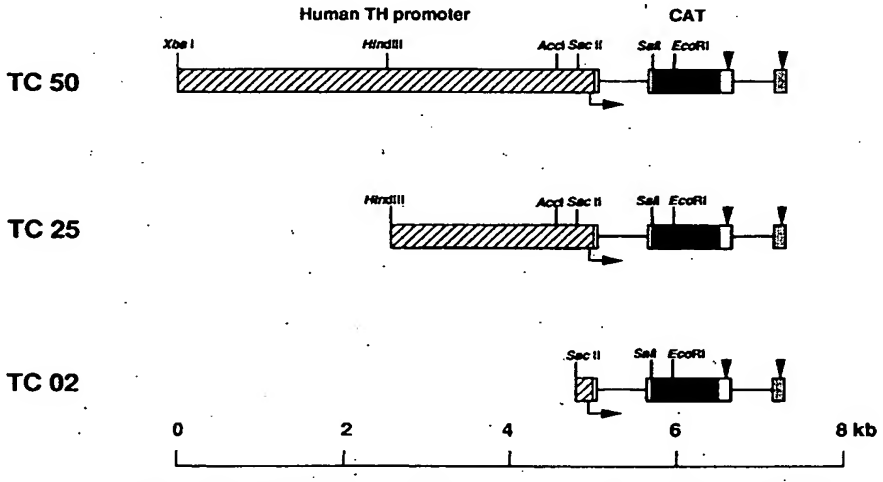

Fig. 1. Structures of the human TH promoter-CAT fusion genes. The TC50 gene consists of the 5-kb human TH 5'-flanking sequence, the second intron of the rabbit β-globin gene, the CAT reporter gene, and the polyadenylation signals of the rabbit β-globin and the SV 40 early genes. The TC25 gene and the TC 02 gene are truncated at the *Hin*dIII and the *Sac*II restriction sites, respectively, to generate the 2.5-kb and 214-bp DNA fragments from the TH gene. The hatched rectangles represent the human 5'-flanking sequences. The solid rectangles represent the CAT coding sequences. The open and dotted rectangles represent the rabbit β-globin and SV40 sequences, respectively. Arrows represent the transcription initiation sites and arrow heads represent the polyadenylation signals. The TC 50 gene or the 1.2-kb *Acc*I–*Sal*I DNA fragment was used as a probe for Southern hybridization.

of the three transgenes, CAT expression in various tissues from the TC 50-2, TC 50-10, TC 25-4, TC 02-3 and TC 02-4 mice were examined. Since each assay contained the same amount of tissue protein, CAT activity in each tissue reflects the relative expression level. The TC 50-2 mouse expressed CAT activity in brain, adrenal gland, and spinal cord, which contain CAnergic neurons or chromaffin cells, and in thymus,

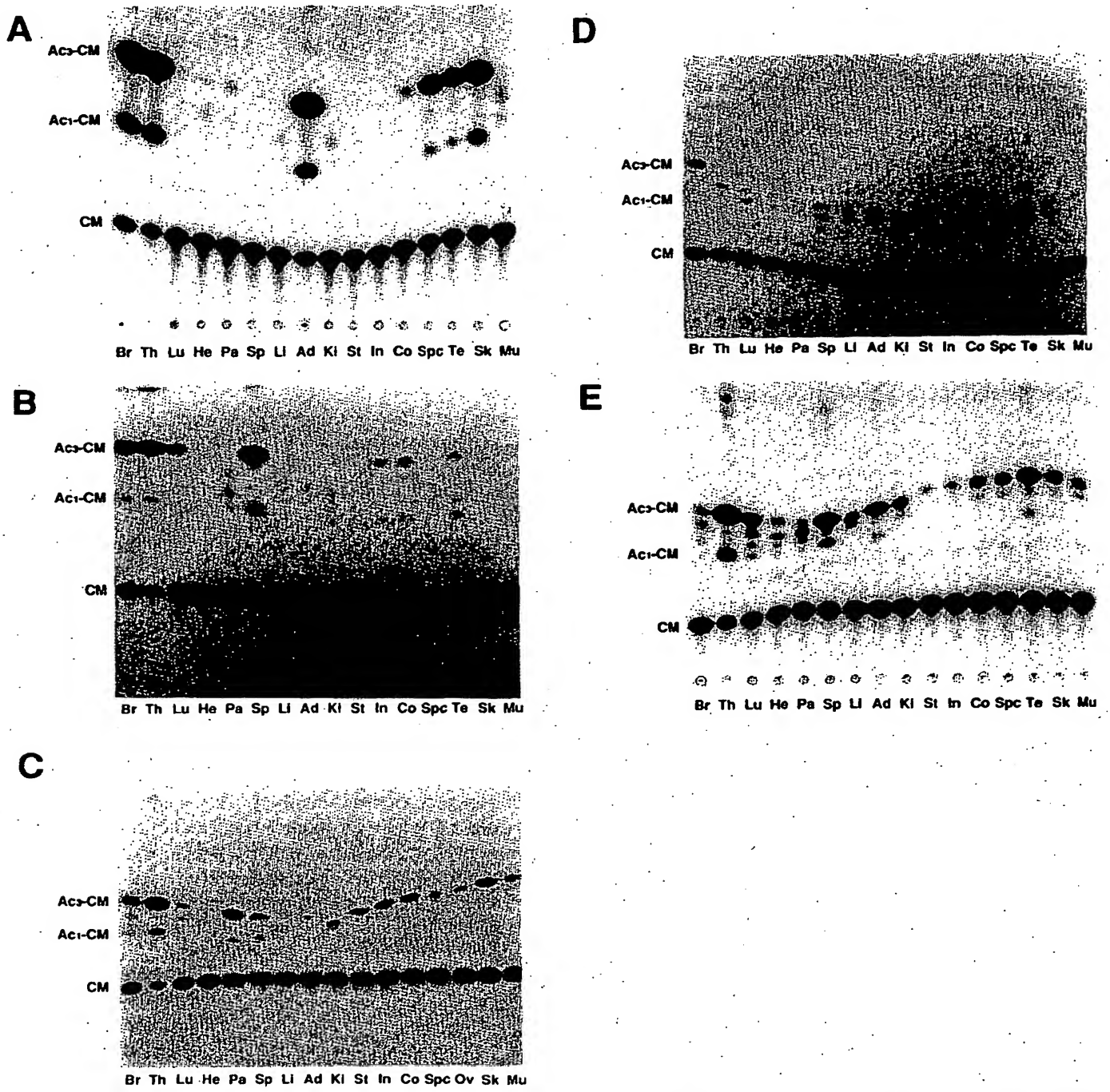

Fig. 2. Expression patterns of the human TH promoter-CAT fusion genes. The CAT expression in various tissues was examined in the transgenic line expressing CAT activity in the brain. CAT assay was carried out with 50 μg protein from tissue extracts of the TC 50-2 (A), the TC 50-10 (B), the TC 25-4 (C), the TC 02-3 (D) and the TC 02-4 mice (E). The unacetylated (CM) and acetylated (Ac1-CM, Ac3-CM) forms of chloramphenicol are shown. Br, brain; Th, thymus; Lu, lung; He, heart; Pa, pancreas; Sp, spleen; Li, liver; Ad, adrenal gland; Ki, kidney; St, stomach; In, intestine; Co, colon; Spc, spinal cord; Te, testis; Sk, skin; Mu, muscle.

testis, and skin. Low activity was found in pancreas and colon (Fig. 2A). The TC 50-10 mouse expressed CAT activity in brain, thymus, spleen, and low activity in lung, intestine, colon and testis (Fig. 2B). In both TC 50-2 and TC 50-10 lines, the CAT activity was at the highest level in brain among all organs examined. Both transgenic lines commonly exhibited ectopic expression in thymus, colon, and testis. The TC 25-4 mouse expressed moderate CAT activity in brain, thymus, pancreas, stomach, intestine, colon, skin, and muscle, and very low activity in lung, spleen, adrenal gland, spinal cord, and ovary (Fig. 2C). The TC 02-3 mouse expressed low CAT activity in the brain and testis (Fig. 2D), and the TC 02-4 mouse expressed moderate CAT activity in thymus, spleen, adrenal gland, and testis, and low activity in all other organs (Fig. 2E). The CAT activity in the brains of TC 25-4 and TC 02-4 mice was lower than that in other organs, such as thymus. This result indicates that the TC 50 gene promotes gene expression in the brain more frequently and intensively than the TC 25 and the TC 02 genes. Two TC 50 lines exhibited ectopic CAT expression in thymus, colon, and testis, and the two TC 02 lines showed ectopic expression in the testis. The ectopic expression of at least two transgenes, the TC 50 and the TC 02, in non-CAnergic tissues could be attributed to their transgene constructs.

TABLE I

CAT activity and CAT immunoreactivity in the brains of transgenic mice carrying the TC 50, TC 25, and TC 02 chimeric gene constructs

[a] These founders died before sexual maturation. N.D., not determined.

Founders	*Copy number*	*Germline transmission*	*CAT activity*	*CAT immunoreactivity*
TC 50 *transgenic*				
TC 50-1	20	+	N.D.	+
TC 50-2	30	+	+	+
TC 50-3	1	+	+	N.D.
TC 50-4	1	+	+	N.D.
TC 50-5	1	+	N.D.	N.D.
TC 50-6 [a]	10		+	N.D.
TC 50-7 [a]	8		+	N.D.
TC 50-8	1	−	+	N.D.
TC 50-9 [a]	20		+	N.D.
TC 50-10	50	+	+	+
TC 50-11 [a]	50		+	N.D.
TC 25 *transgenic*				
TC 25-1	4	+	−	N.D.
TC 25-2	10	+	−	N.D.
TC 25-3	1	+	−	N.D.
TC 25-4	10	−	+	N.D.
TC 25-5	10	+	−	N.D.
TC 25-6	10	+	+	+
TC 25-7	1	+	N.D.	−
TC 25-8	5	+	N.D.	+
TC 02 *transgenic*				
TC 02-1	5	+	+	N.D.
TC 02-2	2	+	−	N.D.
TC 02-3	1	−	+	N.D.
TC 02-4	5	+	+	N.D.
TC 02-5	1	+	−	N.D.
TC 02-6	10	+	N.D.	−
TC 02-7	8	+	+	−
TC 02-8	30	+	N.D.	−
TC 02-9	50	−	−	N.D.
TC 02-10	4	+	+	N.D.
TC 02-11	10	+	+	−

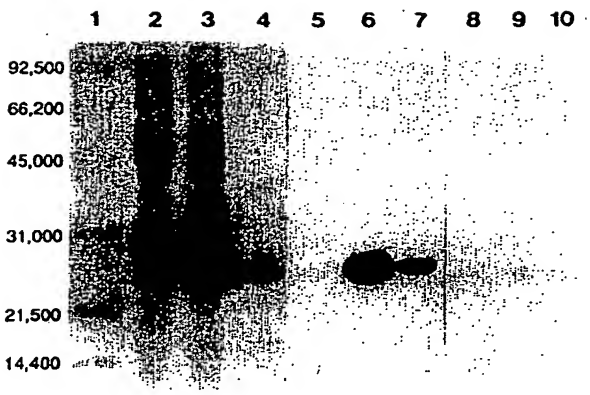

Fig. 3. Antigen specificity of the anti-CAT serum revealed by immunoblot analysis. Extracts from cells transformed with the CAT expression vector and from non-transformed cells were subjected to immunoblot analysis. Lane 1, molecular weight markers; lanes 2, 5, and 8, extract from non-transformed cells; lanes 3, 6 and 9, extract from transformed cells; lanes 4, 7 and 10, purified CAT protein. Protein bands were visualized by Coommasie Brilliant blue staining (lanes 1–4). The CAT-immunoreactive bands were detected with the anti-CAT serum and ^{125}I-labeled protein A (lanes 5–7). The negative control employed preimmune serum instead of the anti-CAT serum (lanes 8–10).

Production of anti-CAT antibody

To elucidate precisely the cell types expressing the reporter gene in the transgenic brain and adrenal gland, we produced anti-CAT antibody in order to perform immunocytochemical analysis. A plasmid vector consisting of a tac promoter and CAT protein coding sequences was constructed for expression of CAT in *E. coli*. CAT protein was purified from the cells transformed with the plasmid according to the method described by Shaw[33]. Rabbits were subsequently immunized with the purified CAT, and anti-CAT antiserum was obtained after total exsanguination. To determine antigen specificity of the antiserum obtained, immunoblotting was performed (Fig. 3). With the anti-CAT serum, the immunoreactivity was detected in the extract from the CAT-producing cells (lane 6) at the same molecular size as the purified CAT (lane 7), but not in the extract from non-transformed cells (lane 5). No immunoreactivity was found with the pre-immune rabbit serum (lanes 8–10). This result indicates that the

antiserum can be used as a specific anti-CAT antibody for immunocytochemical analysis.

Immunocytochemical analysis of the TC 50 transgenic mice

The transgenic lines expressing CAT activity in the brain were subjected to immunocytochemical analysis (summarized in Table II). We examined at least two transgenic littermates of each line by the immunocytochemical analysis and obtained consistent results independently. Of the TC 50 transgenic lines, adult mice of the TC 50-2 line were analyzed at 3–5 months of age. Figs. 4 and 5 show the sections of midbrain, pons, medulla oblongata, and adrenal gland from transgenic (Figs. 4A,B,D–G and 5A–F) and nontransgenic mice (Figs. 4C and 5G). The sections were stained with either anti-CAT antibody (Figs. 4A,C,D,F and 5A,C,E,G) or anti-TH antibody (Figs. 4B,E,G and 5B,D,F). The distribution of mouse TH immunoreactivity in the transgenic brain and adrenal gland was not distinguishable from that in nontransgenic mice (data not shown). The expression pattern of CAT-immunoreactivity was compared directly with that of the mouse TH immunoreactivity in adjacent sections. The CAT protein was detected in specific regions of the transgenic brain (Figs. 4A,D,F and 5A,C), but not in the nontransgenic brain (Fig. 4C). The localization of CAT immunoreactivity was compared with TH immunoreactivity in both CAnergic regions, including DA neuron-, NE neuron- and EPI neuron-rich regions, and non-CAnergic regions (Table II).

The coronal sections of midbrain are shown in Fig. 4A–C. CAT immunoreactivity was localized specifically in the substantia nigra and ventral tegmental area, which are typical DA neuron-rich regions (Fig. 4A). Apparently the number of CAT-immunoreactive cells (Fig. 4A) was lower than that of the TH-immunoreactive cells (Fig. 4B) in these regions. The intensity of CAT immunoreactivity cannot be compared with that of TH immunoreactivity because there

TABLE II

Spatial distribution of CAT and TH immunoreactivities in the brain and adrenal gland of transgenic and non-transgenic adult mice

The signs represent the abundance of CAT and TH immunoreactivities in the different brain regions: + + +, high; + +, moderate; +, low; and −, none. CAnergic regions are classified as described by Hökfelt et al.[10]. [a] The hTH-1 mice were generated and analyzed in previous studies[13,27]. Abbreviations in parentheses represent CAnergic neuron subtypes: DA, dopamine; NE, norepinephrine; and EPI, epinephrine.

Regions	*Transgenic mice*				*Nontransgenic mice*	
	TC 50-2	*TC 25-6*	*TC 02-6*	*hTH-1* [a]		
	CAT	*CAT*	*CAT*	*TH*	*CAT*	*TH*
CAnergic region						
A16 (DA)	−	−	−	+ +	−	+ +
A15–A11 (DA)	+	−	−	+ +	−	+ +
A10 (DA)	+ +	−	−	+ + +	−	+ + +
A9 (DA)	+	−	−	+ + +	−	+ + +
A6 (NE)	+	−	−	+ + +	−	+ +
A5 (NE)	+	−	−	+	−	+
A2/C2 (EPI)	+	−	−	+ +	−	+
A1/C1 (EPI)	+	−	−	+	−	+
Area postrema (NE)	+	−	−	+ +	−	+
Adrenal medulla	+	−	−	+ + +	−	+ +
Non-CAnergic region						
Accessory olfactory bulb	+	−	−	+	−	−
Anterior olfactory nucleus	+	+	−	+ +	−	−
Septum	+ +	−	−	+	−	−
N. accumbens	+ +	−	−	+	−	−
Pyriform cortex	+	−	−	+ + +	−	−
Entorhinal cortex	+	+	−	+ + +	−	−
Hippocampus	+	+ +	−	+ +	−	−
Corporis callosi	+	+	−	+	−	−
Caudoputamen	+ +	−	−	+	−	−
St. terminalis	+	−	−	+	−	−
N. suprachiasmaticus	+	−	−	+	−	−
Amygdaloid complex	+ + +	+	−	+ +	−	−
Lateral habenula	+	−	−	+ +	−	−
Mammillary body	+	−	−	+ +	−	−
Inferior colliculus	+	−	−	+ +	−	−
N. parabrachialis	+	−	−	+	−	−
Parasubiculum	+	+	−	+	−	−
Temporal cortex	+	+	−	+ +	−	−

may be difference in immunoreactivity between anti-CAT and anti-TH antibodies. The CAT immunoreactive cells (Fig. 4A) were neuron-like cells that had morphologic characters of a typical CA neuron, such as multiple dendrites, a long axon, and varicosities. As a typical NE neuron-rich region, the pons sections are shown in Fig. 4D,E. The CAT immunoreactivity was expressed specifically in the locus ceruleus (Fig. 4D), but the number of CAT-immunoreactive cells was significantly less than that of TH-immunorcactive cells

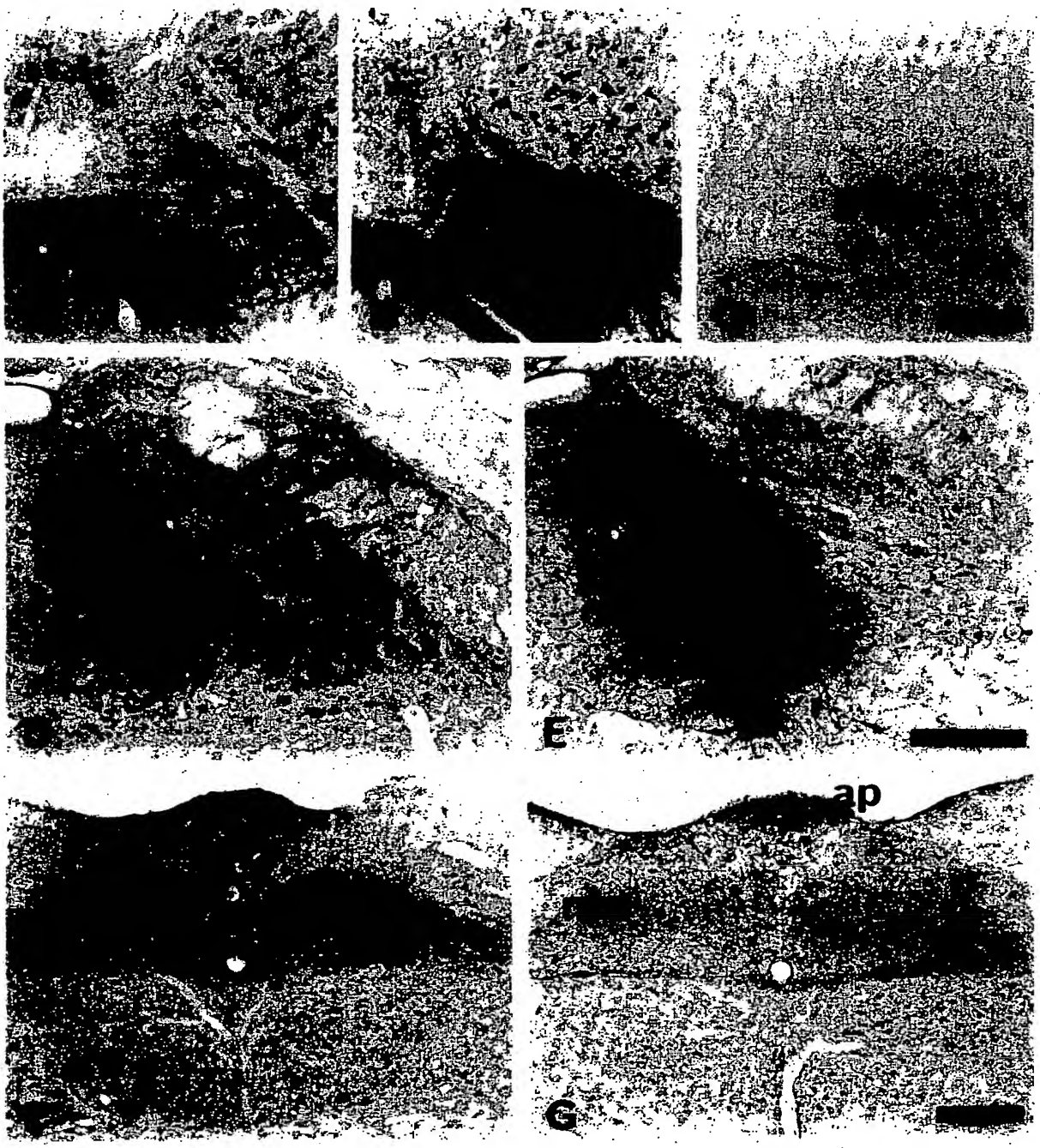

Fig. 4. Immunocytochemical localization of CAT (A, C, D and F) and TH (B, E and G) in transgenic (A, B, D-G) and nontransgenic (C) mice. Sections (40 μm) of brain and adrenal gland were prepared from transgenic and nontransgenic mice and processed for immunocytochemistry of CAT and TH by the peroxidase–antiperoxidase method. Light microscopic photographs of coronal sections of ventral tegmental area (VTA) region (A–C), locus ceruleus (lc), nucleus parabrachialis (npb) (D, E), and dorsal part of the medulla oblongata, nucleus tractus solitarius (nts), area postrema (ap) (F, G). Bars = 200 μm.

(Fig. 4E). The anti-CAT-positive cells in this region had characteristics similar to those of NE neurons. Fig. 4F,G shows sections of the medulla oblongata, which contain both EPI and NE neurons. The CAT immunoreactivity was localized in the nucleus tractus solitarius and in the area postrema, which are typical EPI neuron- and NE neuron-rich regions, respectively. The number of CAT-immunoreactive cells in the medulla oblongata was larger than that in the midbrain or pons sections, and comparable to that of TH immunoreactive ones. The results suggest that CAT immunoreactive cells vary in number among three CAnergic regions, and that the TC 50 gene promotes CAT expression more intensively in EPI neurons than in DA or NE neurons.

CAT immunoreactivity was also found in non-CAnergic regions, such as septum (Fig. 5A) and amygdaloid complex (Fig. 5C, D), as shown in Table II. More interestingly, neuron-like cells with CAT immunoreactivity were detected in the caudate nucleus and putamen (Fig. 5A), which are regions typically containing many nerve terminals and fibers of DA neurons, but no DA neuron cell bodies in the adult animal (Fig. 5B).

In the transgenic adrenal glands, CAT immunoreactivity was expressed only in medullary cells (Fig. 5E).

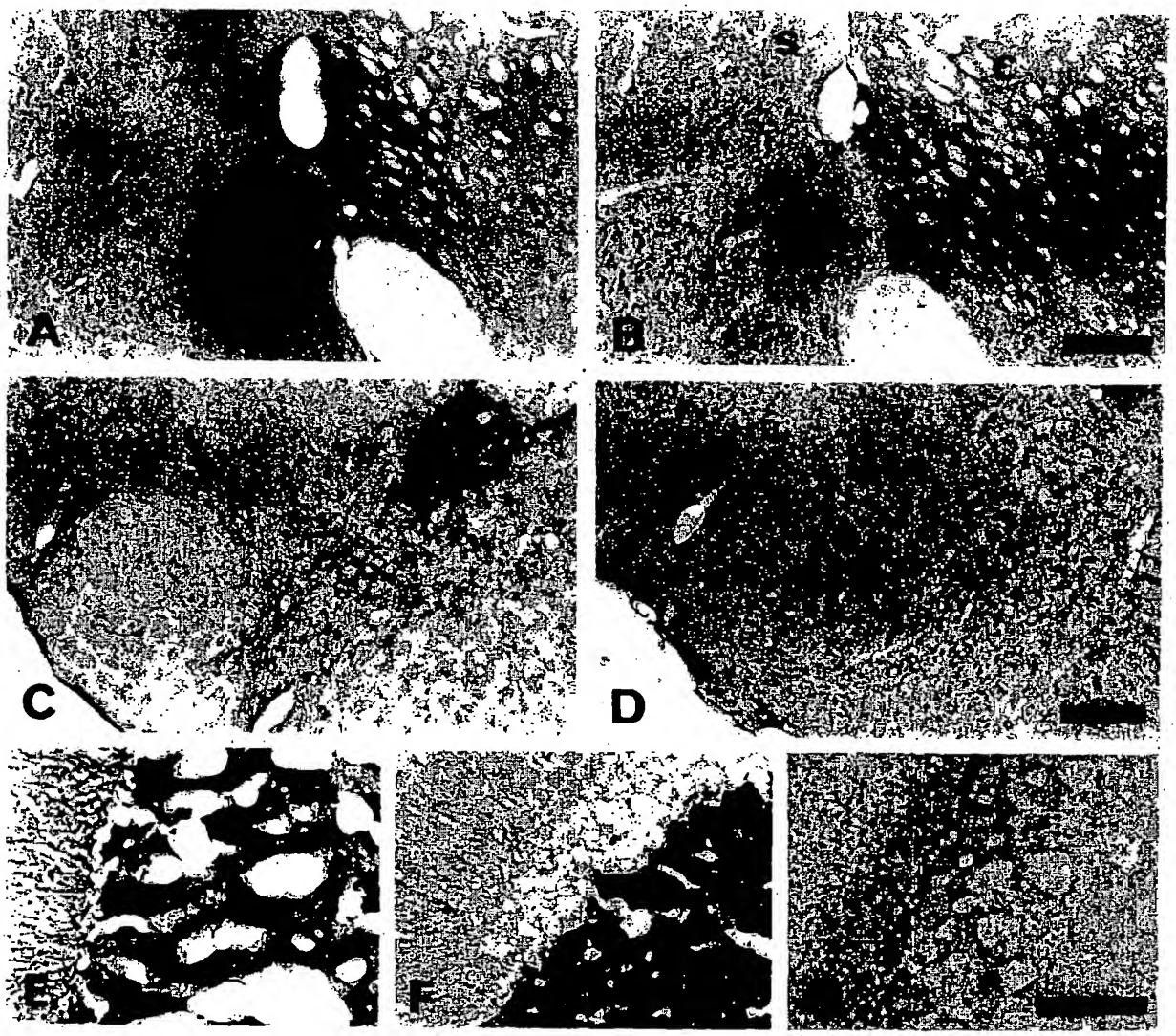

Fig. 5. Immunocytochemical localization of CAT (A, C, E, G) and TH (B, D, F) in transgenic (A-F) and non-transgenic (G) mice. Light microscopic photographs of coronal sections of septum (s), nucleus accumbens (a) and caudoputamen (c) (A, B), amygdaloid complex (ac) (C, D), and adrenal gland (E–G). CAT immunoreactivity is demonstrated in nucleus accumbens, septum, caudoputamen, and amygdaloid complex of only transgenic brain. TH immunoreactivity is usually not expressed in these areas (B, D) in adult brain. Very weak CAT immunoreactivity is observed in some of the adrenal medullary cells (E). Bars = 200 μm.

On the other hand, no CAT immunoreactivity was found in nontransgenic adrenal glands (Fig. 5G). The intensity of CAT immunoreactivity (Fig. 5E) in each medullary cell was uneven and varied, and the expression pattern was similar to that of TH immunoreactivity (Fig. 5F).

Subcellular localization of CAT immunoreactivity in the ventral tegmental area of the midbrain, a typical CAnergic region, was analyzed by immuno-electron microscopy. The apparently discrete, spherical cytoplasmic localization of CAT immunoreactivity was observed in neurons (Fig. 6).

We analyzed two other transgenic lines to examine whether chromosomal integration sites of the transgene would affect its expression. The TC 50-1 and TC 50-10 mice were subjected to immunocytochemical analysis as well, and similar results as described above were obtained (data not shown). Therefore, the pattern of TC 50 expression appears due to its transgene construct and not to some chromosomal position effect. The results indicate that in the brain the 5.0 kb of the 5'-flanking sequence drives expression in typical CAnergic regions but also atypical expression in non-CAnergic regions.

Immunocytochemical analysis of the TC 25 and TC 02 transgenic mice

Among the TC 25 transgenic mice, the TC 25-6 mice were immunocytochemically analyzed. In the brain, CAT immunoreactivity was localized in the non-CAnergic regions, which were anterior olfactory nucleus, entorhinal cortex, hippocampus, corporis callosi, amygdaloid complex, parasubiculum and temporal cortex (Table II). No CAT immunoreactivity was detected in the CAnergic regions, nor in the adrenal gland (data not shown). Additionally, the TC 25-7 and TC 25-8 mice were analyzed, as well. CAT immunoreactivity was detected in the brain of the TC 25-8 mice, and the distribution was consistent with that found in the TC 25-6 mice, but the TC 25-7 mice did not exhibit CAT expression in the brain (data not shown).

Four lines of the TC 02 transgenic mice, TC 02-6, TC 02-7, TC 02-8, and TC 02-11, were subjected to immunocytochemical analysis. No CAT immunoreactivity was observed in any region of TC 02-6 (Table II) or in any of the other TC 02 transgenic lines (data not shown). The TC 25 mice expressed CAT immunoreactivity in some non-CAnergic regions of the brain, but not in the adrenal gland, whereas the TC 02 mice

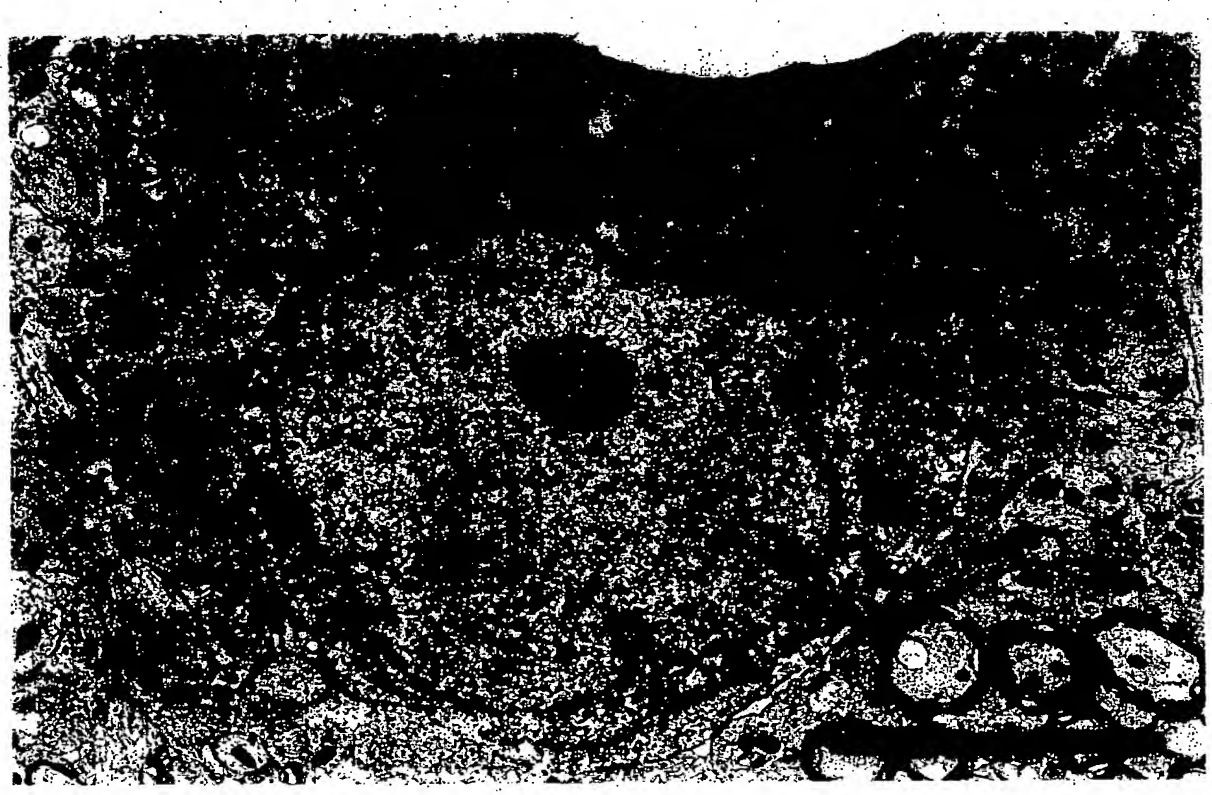

Fig. 6. Ultrastructure of CAT-positive perikarya in ventral tegmental area (VTA) at adult stage of transgenic mouse shows CAT-labeled cytoplasm. The staining is not localized in the nucleus, Golgi apparatus, or mitochondria. Bar = 5 μm.

expressed CAT immunoreactivity neither in the brain nor in the adrenal gland. This result is in sharp contrast to our previous findings obtained using the human TH gene which contained 2.5 kb of the 5′-flanking sequence, as well as all exon–intron sequences and 0.5 kb of the 3′-flanking region[13], and indicates that the 2.5 kb of the human TH 5′-flanking sequence alone is not sufficient to drive CAnergic cell-specific gene expression. Since the TC 02 mice expressed CAT immunoreactivity neither in the brain nor in the adrenal gland, 0.2-kb TH 5′-fragment cannot function in neuron-specific gene expression. Our data obtained from three transgenic mice, TC 50, TC 25, and TC 02, indicate that the 5.0-kb human TH 5′-flanking fragment can drive gene expression in appropriate CAnergic neurons but lacks *cis*-elements for attenuating ectopic expressions in non-CAnergic neurons and in somatic cells and that the 2.5-kb or 0.2-kb 5′-flanking fragment of the TH gene alone cannot express CAT in CAnergic neurons.

DISCUSSION

Tissue-specific regulation of three transgene constructs analyzed by CAT activity assay

Expression patterns of the TC 50, TC 25 and TC 02 chimeric gene constructs were first analyzed by CAT activity assay. All TC 50 mice examined expressed CAT activity in the brain, whereas the TC 25 and the TC 02 mice expressed CAT activity in the brain in two of six lines and in six of nine lines, respectively (Table I). Thus, the TC 50 mice expressed CAT activity in the brain much more frequently than the TC 25 and the TC 02 mice. To examine tissue-specificity of the transgene expression, various tissues from these lines were subjected to CAT assay. The TC 50 mice showed CAT activity in the brain and the adrenal glands, where CAnergic, TH-containing cells exist. They also exhibited ectopic expression in non-CAnergic tissues. In the TC 50-2 mice, ectopic CAT expression was observed in thymus, testis, and skin; whereas in the TC 50-10 mice ectopic expression was found in thymus, spleen, lung, intestine, colon, and testis. The TC 25-4 mice also showed ectopic expression in thymus, lung, pancreas, spleen, stomach, intestine, colon, spinal cord, ovary, skin, and muscle in addition to brain and adrenal gland. The TC 02-3 mouse showed CAT activity in brain and testis, and the TC 02-4 mouse, in all organs examined. The distribution of CAT-expressing tissues varied from line to line, and this may reflect chromosomal position effects that are commonly observed in transgenic mice[31]. However, two TC 50 lines commonly exhibited ectopic CAT expression in thymus, colon, and testis; and two TC 02 lines showed ectopic expression in testis. Therefore, the ectopic expression in these tissues is likely due to the TC 50 and TC 02 gene constructs, rather than the effects of chromosomal integration.

The TC 50-2 and TC 50-10 mice showed the highest CAT expression in the brain among all tissues examined. CAT activities in the brain of the TC 25 and TC 02 mice were lower than those in other organs. As we reported previously[13,39], hTH-1 mice carrying the entire human TH gene including 2.5 kb of the 5′-flanking region, the entire exon–intron sequence, and 0.5 kb of the 3′-flanking region exhibited high-level and tissue-specific expression of human TH in the brain and adrenal glands. These results suggest that the tissue-specific regulation of TH gene expression might be due to multiple regulatory mechanisms, including some suppressive elements as well as positive elements. A recent report has described a similar regulatory mechanism; the 4-kb SCG 10 gene promoter has been shown to drive correct expression in the brain and adrenal glands of transgenic mice, and deletion of the distal regulatory domain of the transgene has yielded deregulated ectopic expression, although expression remained highest in the brain[38]. The 5′-flanking region of the TH gene alone would be ineffective in suppressing the ectopic CAT expression, and some suppressive elements might reside in the exon–intron structure or the 3′-flanking region.

Several sequences homologous to some regulatory elements, such as GC-box, AP-1 binding site, and cAMP response element, are located within 0.2 kb upstream from the transcription start site of the TH gene[14,17,29]. In fact, six TC 02 lines expressed CAT activity in brain. However, CAT immunoreactivity was not detected in any mouse brains of the TC 02 lines (Table I). One possible explanation is that although the TC 02 gene may have general promoter activity in numerous tissues, CAT expression driven by the TC 02 gene may be located too diffusely to be detectable by anti-CAT antibody.

Transgene expression in catecholaminergic regions analyzed by CAT immunoreactivity

The transgenic lines expressing CAT activity in the brain were analyzed immunocytochemically. In three lines of the TC 50 mice examined, CAT immunoreactivity was detected in neuronal somas, dendrites, and fibers of CAnergic regions, including DA, NE, and EPI neurons, and in chromaffin cells of adrenal glands. Ultrastructural analysis revealed that the CAT immunoreactivity was detected in cytoplasm of neurons. However, CAT immunoreactivity in CAnergic regions

was found in transgenic brains of neither the TC 25 mice nor the TC 02 mice. This finding suggests that the 5.0 kb of TH 5′-flanking region could drive gene expression in appropriate CAnergic regions. In a previous study, hTH-1 transgenic mice carrying the entire human TH gene, including 2.5 kb of the 5′-flanking region, 8 kb of the exon–intron structure, and 0.5 kb of the 3′-flanking region, expressed TH immunoreactivity highly and tissue-specifically in typical CAnergic regions[13,39]. Thus, we concluded that the transgene contained CAnergic-neuron specific elements. The 5′-flanking fragment of the TC 25 gene corresponds to the entire 5′-flanking segment present in the above human TH gene, but this 2.5-kb 5′-fragment alone could not express CAT in CAnergic regions of TC 25 transgenic mice. These results suggest that the 5.0 kb of the TH 5′-flanking region might lack some elements suppressing ectopic expression in non-CAnergic tissues but could drive gene expression in appropriate CAnergic regions, and that the 2.5-kb TH 5′-fragment alone might have promoter activity in the brain, but might not function specifically in CAnergic neurons without the inclusion of another CAnergic neuron-specific element(s), and it is suggested that in addition to the 5′-flanking fragment the exon–intron structure and/or the 3′-flanking region of the TH gene could function in the CAnergic neuron-specific gene expression.

In the TC 50 transgenic mice, the number and the staining intensity of CAT-immunopositive cells in CAnergic regions, such as ventral tegmental area (Fig. 4A,B), locus ceruleus (Fig. 4D,E), and adrenal medulla (Fig. 5E,F), were lower than those of TH-immunopositive cells. Since there may be differences in immunoreactivity between anti-CAT and anti-TH antibodies, the intensity of the CAT immunoreactivity cannot be directly compared with that of TH immunoreactivity. Apparently, the TC 50 mice expressed CAT in some of the endogenous mouse TH-containing cells. On the other hand, the hTH-1 mice carrying the entire human TH gene exhibited high-level expression of human TH in the CAnergic regions[13,39]. There are several possible explanations for small numbers of CAT-immunoreactive cells in the TC 50-2 mice. First, the heterologous gene, CAT, may not behave equally to endogenous TH in translational efficiency, molecular stability, or subcellular localization; and hence the quantitative expression of the CAT gene may be less than that of TH. Second, the regulatory element in the 5′-flanking region might be insufficient for the quantitative regulation of the transgene expression, and the *cis*-element for the quantitative regulation may reside in the exon–intron structure or the 3′-flanking region of the human TH gene. The recent report that an enhancer-like activity resides in the 3′-flanking region of the human TH gene as judged by transient transfection assay in cultured cells[4] supports the second possibility.

Atypical transgene expression in non-catecholaminergic neurons analyzed by CAT immunoreactivity

The TC 50-2 mice expressed CAT immunoreactivity in CAnergic neurons as well as in non-CAnergic neurons (Fig. 5A,C and Table II), and the TC 25-6 (Table II) and TC 25-8 mice expressed CAT immunoreactivity only in some non-CAnergic neurons. Since CAT expression in non-CAnergic neurons was observed consistently in multiple lines of the TC 50 and TC 25 mice, this expression pattern should be due to the characters of the two transgene constructs, and not to the chromosomal insertion sites. Atypical transgene expression in sensory systems was also observed in hTH-1 mice carrying the entire human TH gene[13,27] (see Table II). There are two possible explanations for atypical transgene expression in non-CAnergic neurons. First, it could be due to species difference in the regulatory mechanism of TH gene expression. Second, the transgene constructs would be insufficient to suppress the atypical expression in non-CAnergic neurons. Recent papers reported that transient TH expression was observed in developing rodent brain, for example, in the anterior olfactory nucleus[26], inferior colliculus[12], cerebral cortex[1,32], amygdala[37], and cerebellum[9]. Such cells that transiently express TH do not contain other CA-synthesizing enzymes and the functions of these cells have been unclear[1,12,26,37]. The transgenes, TC 50 and TC 25, as well as the entire human TH gene, drove persistent expression in non-CAnergic regions including the cells exhibiting transient TH expression as described above. These gene constructs might be insufficient to attenuate the transgene expression in such non-CAnergic regions in adult-stage mice. There are mouse mutants, tottering and leaner, which exhibit neurologic disorders that result from recessive mutant alleles of a single genetic locus mapped to mouse chromosome 8[36,40]. These animals are deficient in suppressing the normal transient expression of TH in developing Purkinje cells, which indicates the presence of a suppressive regulatory mechanism for TH gene expression[9]. Some additional regulatory elements for suppressing transient expression may be necessary for our transgene constructs to mimic correctly the endogenous TH expression pattern. A recent report has described that the DBH gene promoter directed *E. coli lacZ* reporter gene expression in sympathetic and other neurons in adult transgenic mice[23]. We have also found that transgenic mice carrying the human DBH promoter-human PNMT cDNA fusion gene exhibited

transgene expression in CAnergic cells and in some non-CAnergic neurons[15]. The expression patterns of the human TH promoter-CAT constructs in non-CAnergic regions overlap with several sites where the human DBH promoter drives gene expression. These results suggest that the TH and DBH genes may contain some common elements which suppress atypical expression in non-CAnergic neurons.

CONCLUSION

We have demonstrated that the 5.0-kb 5′-flanking region of the human TH gene could drive bacterial CAT reporter gene expression in the CAnergic neurons and adrenal medullary cells of transgenic mice. CAT expression was also observed in some non-CAnergic neurons, including those in several sites where transient TH expression has been reported. The 2.5-kb and 0.2-kb 5′-flanking fragments of the TH gene could not express CAT in CAnergic neurons. We conclude that the 5.0 kb of the human TH 5′-flanking region may lack some elements suppressing ectopic expression in non-CAnergic neurons, and that the exon–intron structure and/or 3′-flanking region of the TH gene may also function in CAnergic neuron-specific expression.

To identify precisely the CAnergic neuron-specific elements, various deletion fragments from the 5′-flanking region, the exon–intron region or the 3′-flanking region fused to the CAT gene should be examined into the transgenic mouse system. This approach would make it possible to characterize the regulatory elements responsible for expression in anatomically definable regions, and also may provide clues to the identity of *trans*-acting factors responsible for CAnergic neuron-specific gene expression.

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The 5′-flanking region of the human dopamine β-hydroxylase gene promotes neuron subtype-specific gene expression in the central nervous system of transgenic mice

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Dopamine β-hydroxylase (DBH, EC 1.14.17.1) catalyzes the conversion of dopamine to norepinephrine, the third step of catecholamine biosynthesis. We have previously created transgenic mice harboring a chimeric gene consisting of the 4-kb DNA fragment of the human DBH gene promoter and the human phenylethanolamine *N*-methyltransferase (PNMT, EC 2.1.1.28) cDNA, to express PNMT in norepinephrine- and epinephrine-producing cells in the brain, sympathetic ganglia, and adrenal medullary chromaffin cells (Kobayashi et al., *Proc. Natl. Acad. Sci. U.S.A.*, 89 (1992) 1631–1635). In this paper, we produced for the first time the antibody that specifically detects human PNMT, but not mouse PNMT, with the synthetic oligopeptide characteristic of the human PNMT sequence, and used this antibody to investigate the cells expressing human PNMT in transgenic mice. Immunohistochemical analysis of transgenic mice showed typical expression of human PNMT immunoreactivity in norepinephrinergic and epinephrinergic neurons in brain, as well as norepinephrine- and epinephrine-producing cells in the adrenal gland, indicating that the 4-kb 5′-flanking region is essential for the tissue-specific expression of the DBH gene. We also detected the ectopic expression in some DBH-immunonegative cells in the olfactory bulb of transgenic mice.

INTRODUCTION

The mammalian brain and sympathetic nerve synthesize 3 different catecholamines as neurotransmitters: dopamine, norepinephrine, and epinephrine; the adrenal medulla also produces norepinephrine and epinephrine as hormones. The catecholaminergic phenotypes are characterized by different gene expression patterns of the 4 catecholamine-synthesizing enzymes, tyrosine hydroxylase (TH, EC 1.14.16.2), aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28), dopamine β-hydroxylase (DBH, EC 1.14.17.1) and phenylethanolamine *N*-methyltransferase (PNMT, EC 2.1.1.28)[4,18]. Dopaminergic neurons express TH and AADC. Norepinephrine-producing cells express TH, AADC, and DBH. Epinephrine-producing cells express all 4 enzymes including PNMT. The functional analysis of each gene promoter is of importance in the investigation of the molecular mechanism of cell type-specific expression in catecholamine-producing cells in the nervous and endocrine systems.

DBH is a monooxygenase that consists of 4 identical subunits of 72 kDa; it catalyzes the conversion of dopamine to norepinephrine[2,21]. This enzyme is localized in norepinephrinergic neurons in various brain regions including the locus ceruleus, ventrolateral metencephalon, and dorsolateral myelencephalon, as well as in epinephrinergic neurons of the nucleus tractus solitarius region[3]. In peripheral tissues, DBH is expressed in sympathetic ganglia and adrenal medullary chromaffin cells. We previously reported molecular cloning of cDNA and genomic DNA encoding human DBH[7]. The human DBH gene consists of 12 exons spanning approximately 23 kb and several transcription regulatory elements are present in its 5′-flanking region. Recently, the bovine[10,23,25] and rat[11] DBH cDNA

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clones were isolated and their nucleotide and deduced amino acid sequences were determined.

In our previous research, we used the 4-kb DNA fragment of the human DBH gene promoter to express human PNMT in transgenic mice, and described that this promoter region can function to drive the transgene expression at least in norepinephrine-producing cells of the brain, superior cervical ganglion (SCG), and adrenal gland[8]. However, we could not verify the transgene expression in epinephrine-producing cells in the brain and adrenal gland, because the anti-bovine PNMT antibody used for immunohistochemistry could not distinguish between human PNMT expression and endogenous mouse PNMT. In this study, we newly produced the antibody that detects human PNMT but not mouse PNMT, by immunization with a synthetic oligopeptide characteristic of the human PNMT sequence. The promoter activity in the 4-kb 5′-flanking region of the human DBH gene in transgenic mice was analyzed with this antibody.

MATERIALS AND METHODS

Transgenic mice

The construction of transgenic mice has been described in our previous paper[8]. The transgene consists of the 5′-flanking region of the human DBH gene (4 kb), the rabbit β-globin second intron, the human PNMT cDNA (1 kb) and the rabbit β-globin, and SV40 early gene polyadenylation signals (see Fig. 1). Out of 8 founder mice generated by pronuclear microinjection, two independent transgenic lines, DPN2-6 and DPN1-3, were analyzed in this study. The numbers of transgene copies in the DPN2-6 and DPN1-3 lines were approximately 80 and 70 per haploid genome, respectively. In each transgenic line, the transgene was integrated in a tandem head-to-tail repeat in a single site of mouse genome, and was stably transmitted to the offspring according to a Mendelian hereditary pattern.

Peptide synthesis and production of anti-peptide antibody

The oligopeptide hPNMT-N (MSGADRSPNAGAAPDSAPG), which corresponds to the 1–19 amino acid residue of human PNMT[5], was synthesized with an Applied Biosystem 430A peptide synthesizer[13]. The synthetic peptide was released from the solid-phase support matrix, and purified by chromatography on a Bio-Gel P4 column. The fractions containing the peptide were identified by amino acid analysis. The synthetic peptide (18 mg) was coupled to 30 mg of keyhole limpet hemocyanin with glutaraldehyde (60 μmol), and the reaction mixture was twice dialyzed against phosphate-buffered saline (PBS). For immunization, the peptide conjugate solution (4 mg/ml) was emulsified with Freund's complete adjuvant in a quantity of 1.5× volume of the antigen solution. Each rabbit received a dose corresponding to 2 mg of the peptide intradermally on the back. Doses of the peptide conjugate corresponding to 1 mg of the peptide in Freund's incomplete adjuvant were then given for booster injection at intervals of 2 weeks by subcutaneous injections. Preimmunized serum was obtained from the same rabbit prior to immunization with the synthetic peptide conjugate.

Western blotting

Adrenal glands removed from adult mice were sonicated in 50 mM Tris-HCl buffer (pH 7.3), centrifuged at 10,000 *g* for 10 min, and the supernatant was used for the following analysis. Protein concentration was determined by the method of Bradford[1]. Proteins (50 μg) were subjected to electrophoresis on a 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel according to the technique of Laemmli[9]. After proteins were transferred to nitrocellulose membranes[24], the membranes were incubated with either anti-bovine PNMT antibody[16] or anti-hPNMT-N antibody at a 1:100 dilution in PBS containing 2% bovine serum albumin (BSA). After washing with PBS containing 0.2% Tween 20, the membranes were incubated with ^{125}I-labeled protein A at a 1:100 dilution in PBS containing 2% BSA. Autoradiography was carried out with a Kodak XAR film.

Immunohistochemical procedure

Paraformaldehyde-fixed frozen sections of adult mouse tissues were preincubated with 5% normal swine serum, and incubated with either anti-bovine PNMT antibody (1/3000 dilution), anti-hPNMT-N antibody (1/1000 dilution), or anti-DBH-N antibody[15] (1/3000 dilution). After washing with PBS, the frozen sections were incubated with swine anti-rabbit IgG (1/100 dilution) and peroxidase-antiperoxidase complex (1/200 dilution). Free floating sections were developed with diaminobenzidine tetrahydrochloride as a chromogen.

RESULTS

Production of the antibody against the human PNMT synthetic peptide

Fig. 1 shows the strategy to produce the antibody specific to human PNMT. We have previously reported on the isolation and characterization of the mouse

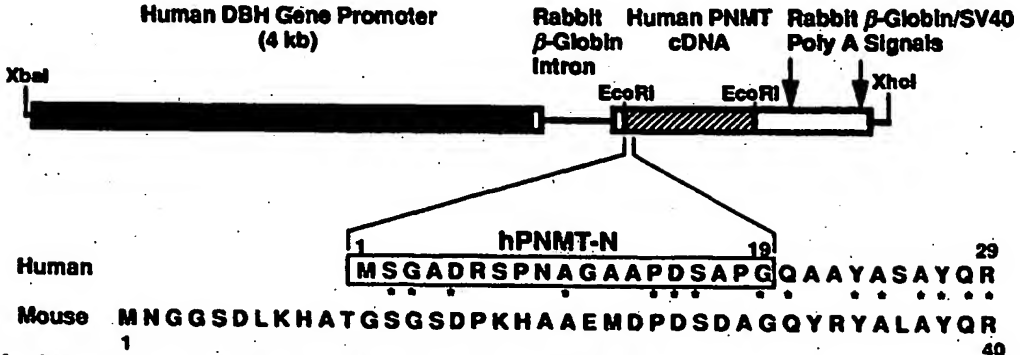

Fig. 1. Strategy for the production of the antibody to detect specifically human PNMT in transgenic mice. Comparison of the *N*-terminal amino acid sequences of human and mouse PNMT is shown beneath the transgene structure. The amino acids identical between two sequences are indicated by asterisks. The synthetic peptide (amino acid residues 1–19 of human PNMT sequence), hPNMT-N, are boxed. The amino acid sequences are derived from the data reported by Morita et al.[14].

PNMT gene, and showed that the existence of 23-bp direct repeat sequences in the 5'-terminal region of the mouse PNMT gene causes the major difference in the *N*-terminal amino acid sequence, as compared to human PNMT[14]. In the *N*-terminus, mouse PNMT was longer than the human enzyme by an extra 11 amino acid residue, and the *N*-terminal region of human PNMT did not show a significant homology to the corresponding region of mouse PNMT. Therefore, we selected 19 amino acids of the human PNMT sequence (amino acid residue 1–19), designated as hPNMT-N, to synthesize the oligopeptide for production of the antibody that differentially detects human PNMT protein. The antibody against the synthetic oligopeptide-hemocyanin conjugate was prepared as described in Materials and Methods.

To examine the specificity of the anti-hPNMT-N antibody, Western blotting was carried out with tissue extracts prepared from adrenal glands of DPN2-6 transgenic and non-transgenic control mice (Fig. 2). Since DPN2-6 transgenic adrenal gland expresses human PNMT in addition to endogenous mouse PNMT[8], we used this transgenic mouse tissue as a positive control containing human PNMT protein. Anti-bovine PNMT antibody cross-reacted with mouse PNMT with a molecular weight of 32 kDa (lane 1). In transgenic mice (lane 2), the PNMT band strongly stained with the same antibody, about 10-fold stronger than in non-transgenic control mice, which confirms the additional expression of human PNMT in transgenic adrenal gland. On the other hand, anti-hPNMT-N antibody stained the PNMT band in transgenic adrenal gland (lane 4), but not in non-transgenic adrenal gland (lane 3), indicating that anti-hPNMT-N antibody can specifically detect human PNMT.

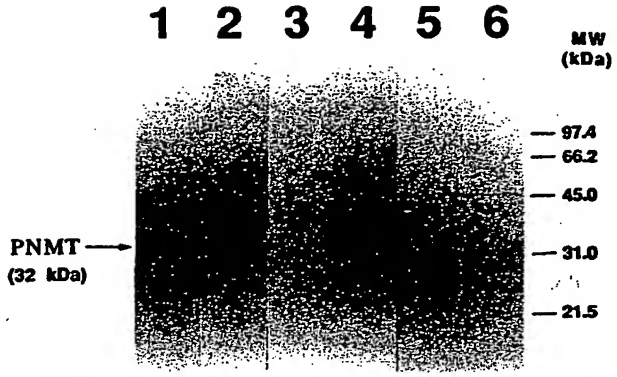

Fig. 2. Western blotting of the mouse adrenal gland extracts. Proteins (50 μg) prepared from adrenal glands of non-transgenic control mice (lanes 1, 3 and 5) and DPN2-6 transgenic mice (lanes 2, 4 and 6) were subjected to SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. The membranes were incubated with either anti-bovine PNMT antibody (lanes 1 and 2), anti-hPNMT-N antibody (lanes 3 and 4), or preimmunized antiserum (lanes 5 and 6). Molecular weight standards: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa) and soybean trypsin inhibitor (21.5 kDa). The PNMT band with molecular weight of 32.0 kDa is indicated by an arrow. The extra bands above the PNMT bands (lanes 1 and 2) are proteins recognized non-specifically by anti-bovine PNMT antibody.

Immunohistochemical analysis of the human PNMT expression in DPN transgenic mice

The expression of transgene products in two independent transgenic lines was analyzed with the immunohistochemical procedure using anti-hPNMT-N antibody. This antibody did not detect any immunoreactivity in the brain and adrenal gland of non-transgenic control mice (data not shown). The human PNMT immunoreactivity was expressed in various brain regions and adrenal gland of DPN2-6 transgenic mice. Fig. 3 shows the typical transgene expression in the cells where DBH is normally localized. In norepinephrine neurons in the ventrolateral metencephalon (A5) of transgenic mouse brain, the human PNMT immunoreactivity was observed (Fig. 3A). In the A5 region of non-transgenic mouse brain, norepinephrine neurons were localized by immunohistochemical analysis with anti-DBH-N antibody (Fig. 3C). These norepinephrine neurons were not stained with anti-bovine PNMT antibody (Fig. 3B). The human PNMT immunoreactivity was also detected in epinephrine neurons in the rostral part of the nucleus tractus solitarius (C2) of transgenic brain (Fig. 3D). The location of epinephrine neurons was identified by the existence of both DBH and PNMT enzymes (Figs. 3F and 3E). Moreover, in transgenic mouse brain, the expression of the transgene products was observed in other norepinephrine neurons in the locus ceruleus (A6/A4), and the lateral region of the mesencephalic/metencephalic border (A7), as well as in other epinephrine neurons in C1 and C3 regions. Subsequently, the human PNMT expression in adrenal gland was investigated with the same method. Norepinephrine- and epinephrine-producing cells were detected scattered throughout the non-transgenic adrenal medulla (Figs. 3H, I). As shown in Fig. 3G, anti-hPNMT-N antibody stained almost all cells in trangenic mouse adrenal medulla, indicating the expression of human PNMT in both norepinephrine- and epinephrine-producing cells. The immunohistochemical analysis of DPN1-3 transgenic mice with anti-hPNMT-N antibody showed similar results to the typical expression in the brain norepinephrine and epinephrine neurons, and adrenal medullary chromaffin cells of DPN2-6 transgenic lines (data not shown). Additionally, we observed the transgene ectopic expression in some non-catecholaminergic

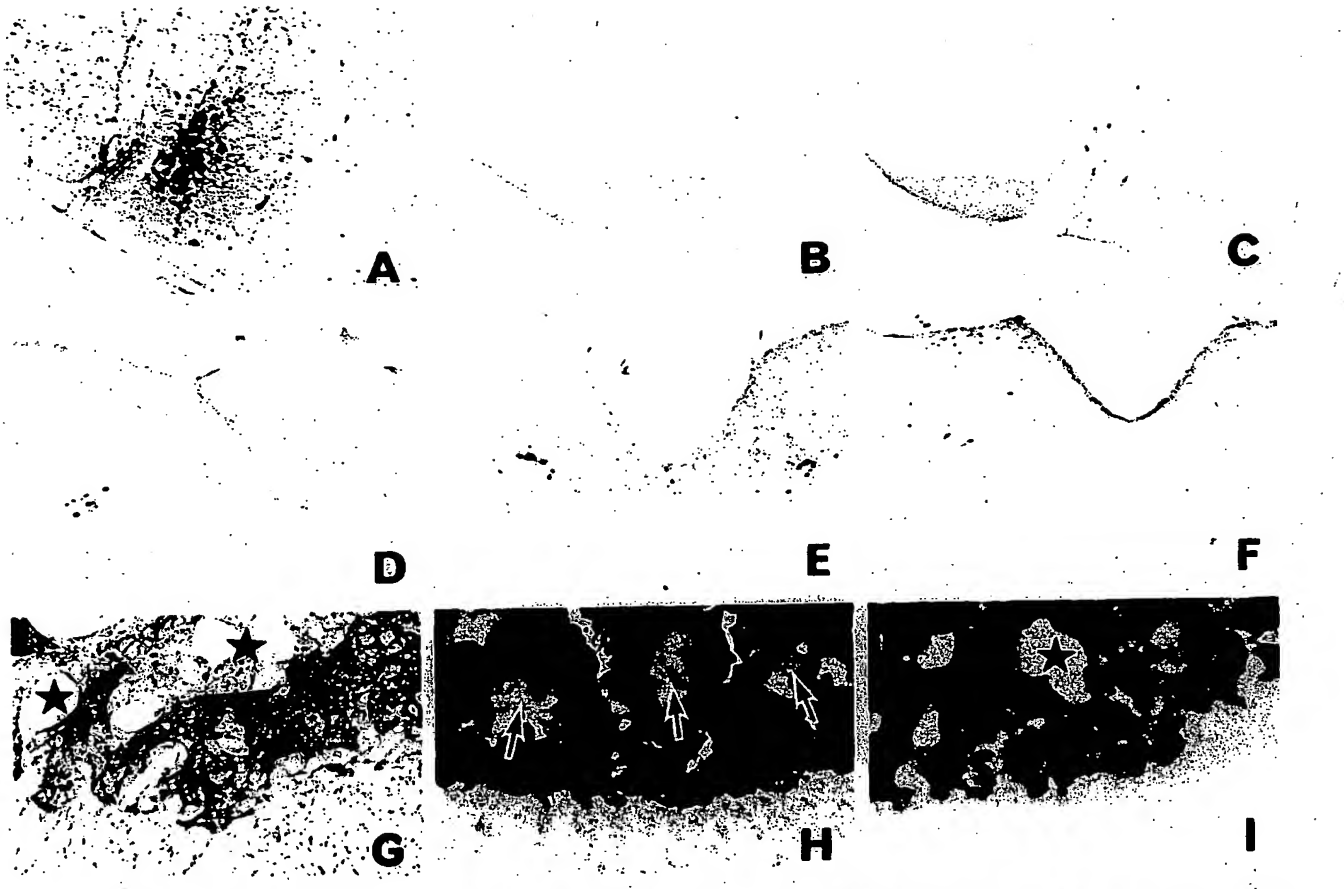

Fig. 3. Typical expression of the human PNMT immunoreactivity in transgenic mice. Frozen sections of DPN2-6 transgenic mice (A, D, G) and non-transgenic control mice (B, C, E, F, H, I) were immunostained with anti-hPNMT-N antibody (A, D, G), anti-bovine PNMT antibody (B, E, H), or anti-DBH-N antibody (C, F, I). Light microscopic photographs of the ventrolateral metencephalon (A5) (A, B, C), the rostral part of the nucleus tractus solitarius (C2) (D, E, F) and adrenal gland (G, H, I). Arrows indicate norepinephrine-producing cells in adrenal gland of non-transgenic mice. Stars show blood vessels. Magnification = (A–F)×55; (G–I)×145.

regions of transgenic mouse brain. In both DPN2-6 and DPN1-3 lines, the human PNMT immunoreactivity was detected in some periglomerular (A16), tufted, mitral, and granule cells in the olfactory bulb (Fig. 4).

DISCUSSION

In this paper, we investigated the expression pattern of the foreign gene products in transgenic mice that contain human PNMT cDNA downstream of the 4-kb segment of human DBH gene promoter. To detect the human PNMT expression by immunohistochemistry, the antibody that specifically recognizes human PNMT protein was generated by immunization with a synthetic oligopeptide, the structure of which was based on the difference between the *N*-terminal amino acid sequences of the human and mouse PNMT enzymes. In transgenic mice, this newly created antibody detected the human PNMT expression in norepinephrine- and epinephrine-producing cells in the brain and adrenal medulla, where the DBH gene is normally expressed. These results indicate that the 4-kb DNA fragment containing the 5′-upstream region of the human DBH gene is sufficient to express the transgene specifically in both norepinephrine- and epinephrine-producing cells.

We observed the ectopic expression of the transgene in some DBH-immunonegative cells. Our previous analysis with immunohistochemistry using anti-bovine PNMT antibody also showed that the PNMT immunoreactivity appeared in some non-catecholaminergic neurons in transgenic mice[8]. The present results confirmed the finding that the transgene products are actually expressed in non-catecholaminergic regions. There are several explanations for the ectopic expression. First, the 4-kb 5′-region of the human DBH gene promoter may lack the *cis*-element necessary to suppress the expression in the ectopic sites. The DBH

Fig. 4. Ectopic expression in the olfactory bulb of transgenic mice. Photograph shows immunostain of the DPN2-6 transgenic brain with anti-hPNMT-N antibody. Periglomerular (PG), tufted (Tu), mitral (Mi) and granule (Gr) cells are indicated. Magnification = ×145.

gene may normally be repressed in the brain regions where the ectopic expression was observed. Second, the sequence diversity in the promoter region between the human and mouse DBH genes, may affect the correct gene expression. Third, the artificial combination of the promoter and the reporter gene may cause the aberrant gene expression in transgenic mice. It is known that transgenic expression in ectopic sites is generated when diverse genetic elements are juxtaposed, as in chimeric genes in transgenic mice[19,20,22]. We also observed the ectopic expression in sensory neurons of the brain of transgenic mice carrying the human TH gene[6,17].

Mercer et al.[12] recently reported that the 5.8-kb region of human DBH gene promoter is sufficient to direct expression of the *E. coli lacZ* gene in transgenic mice to norepinephrine neurons in brain, sympathetic ganglion neurons, and adrenal chromaffin cells. Here, we have described the analysis of the promoter activity in the shorter 4-kb DNA fragment of the human DBH gene promoter. Transgenic mice having the 4-kb region of human DBH promoter showed the typical expression pattern of the transgene, indicating that this promoter region is essential for the tissue-specific expression of the human DBH gene. Also, our analysis showed that two independent transgenic lines express the human PNMT immunoreactivity ectopically in the brain olfactory bulb. In contrast, Mercer et al. never detected the ectopic expression in the olfactory bulb of transgenics having the 5.8-kb DBH promoter, although they observed the expression in some other DBH-immunonegative cells. The presence or absence of the ectopic transgene expression in the olfactory bulb might depend on the difference of the promoter region used in our and their experiments.

In the previous report, we tried to convert catecholamine specificity from norepinephrine to epinephrine phenotype, by the additional expression of PNMT in norepinephrine-producing cells of transgenic mice[8]. We selected the human DBH gene promoter to express human PNMT, because the DBH gene is transcribed in norepinephrine- and epinephrine-producing cells of wild-type mice. The expression of human PNMT in transgenic mice induced the appearance of epinephrine in sympathetic ganglion, and dramatic changes in norepinephrine and epinephrine levels in brain, adrenal gland, and blood, indicating that the additional expression of PNMT in norepinephrine-producing cells can convert these cells to the epinephrine phenotype. Our transgenic mice are important in the investigation of the effects on the neurological and hormonal functions of catecholamines and the regulatory mechanism of adrenergic receptor subtypes, by genetic alteration of catecholamine synthesis. The analysis of the DBH gene promoter provides the basic information on the neurological and physiological investigations of these transgenic animals.

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Neurobiology

Regulatory region in choline acetyltransferase gene directs developmental and tissue-specific expression in transgenic mice

(development/gene promoter/gene regulation/silencer/spinal cord)

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ABSTRACT **Acetylcholine, one of the main neurotransmitters in the nervous system, is synthesized by the enzyme choline acetyltransferase (ChAT; acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6). The molecular mechanisms controlling the establishment, maintenance, and plasticity of the cholinergic phenotype *in vivo* are largely unknown. A previous report showed that a 3800-bp, but not a 1450-bp, 5′ flanking segment from the rat ChAT gene promoter directed cell type-specific expression of a reporter gene in cholinergic cells *in vitro*. Now we have characterized a distal regulatory region of the ChAT gene that confers cholinergic specificity on a heterologous downstream promoter in a cholinergic cell line and in transgenic mice. A 2342-bp segment from the 5′ flanking region of the ChAT gene behaved as an enhancer in cholinergic cells but as a repressor in noncholinergic cells in an orientation-independent manner. Combined with a heterologous basal promoter, this fragment targeted transgene expression to several cholinergic regions of the central nervous system of transgenic mice, including basal forebrain, cortex, pons, and spinal cord. In eight independent transgenic lines, the pattern of transgene expression paralleled qualitatively and quantitatively that displayed by endogenous ChAT mRNA in various regions of the rat central nervous system. In the lumbar enlargement of the spinal cord, 85–90% of the transgene expression was targeted to the ventral part of the cord, where cholinergic α-motor neurons are located. Transgene expression in the spinal cord was developmentally regulated and responded to nerve injury in a similar way as the endogenous ChAT gene, indicating that the 2342-bp regulatory sequence contains elements controlling the plasticity of the cholinergic phenotype in developing and injured neurons.**

The choice of a specific complement of neurotransmitters is a crucial step in the acquisition of a differentiated phenotype by developing neurons. The molecular mechanisms controlling the establishment and plasticity of neuronal phenotypes are only beginning to be understood, as more regulatory sequences and transcription factors affecting pathways of neuronal differentiation are being characterized. Acetylcholine, one of the main neurotransmitters in the nervous system, is synthesized by choline acetyltransferase (ChAT; EC 2.3.1.6), currently the most specific marker for cholinergic neurons. The most prominent cholinergic system consists of cells associated with cranial nerve nuclei and motor neurons of the spinal cord. In the forebrain, the basal forebrain complex contains the major cholinergic cell bodies. These have been implicated in cognitive functions (1) and in the pathogenesis of Alzheimer disease (2). The levels of ChAT enzyme activity and ChAT mRNA are developmentally regulated in the central nervous system (CNS) (3–5), and *in vivo* and *in vitro* studies have demonstrated that ChAT enzyme activity can be modulated by numerous extracellular effector molecules, including small hormones (6–9), cytokines (10), and growth factors (11–18).

In the rat, the gene encoding ChAT has been shown to span >64 kb and to contain 14 coding exons (19) and several alternatively spliced 5′ noncoding exons (20). Transcription of the rat and mouse ChAT genes appears to initiate at multiple promoters resulting in the production of five different 5′ mRNA sequences (20, 21). Different upstream regions of the ChAT gene have been shown to be necessary for cell type-specific expression of reporter genes in cholinergic cell lines (21–23). However, the molecular mechanisms regulating the establishment, maintenance, and plasticity of the cholinergic phenotype *in vivo* are largely unknown. We previously isolated a 3.8-kb segment from the rat ChAT gene promoter that directs expression of a reporter gene to cholinergic cells *in vitro* (22). Here, we show that a 2342-bp-long segment from this region confers cholinergic specificity on a noncholinergic promoter in an orientation-independent manner and directs correct developmental and tissue-specific expression to cholinergic cells in transgenic mice.

MATERIALS AND METHODS

DNA Cloning. A 2342-bp *Eco*RI–*Hin*dIII fragment from a genomic rat ChAT λEMBL3 clone (22) was isolated and subcloned into the *Sal* I site of pBLCAT$_2$ (24). This placed the 2342-bp fragment upstream of a herpes simplex virus thymidine kinase (*tk*) minimal promoter (spanning from −105 to +51) followed by the bacterial chloramphenicol acetyltransferase (CAT) reporter gene and polyadenylylation sequence from the simian virus 40 small-t-antigen gene.

Cell Culture, Transfection, and CAT Assay. FR3T3 fibroblasts and SN6 cells (25) were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (GIBCO/BRL) at 37°C in a 5% CO_2 atmosphere. SN6 is a cholinergic neuronal cell line derived from a fusion of mouse primary septal neurons with the human neuroblastoma cell line N18TG2 and was kindly provided by David Hammond and Alfred Heller (University of Chicago). Cells grown to ≈70% confluency in 35-mm wells were transfected according to the calcium phosphate method with 1.5 μg of DNA plus 0.5 μg of a reference plasmid, pON260 (26), expressing β-galactosidase as internal control for transfection efficiency. After 48 hr, cells were harvested with phosphate-buffered saline containing 10 mM EDTA, transferred to 250 mM Tris (pH 7.5),

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Abbreviations: CAT, chloramphenicol acetyltransferase; ChAT, choline acetyltransferase; CNS, central nervous system; RT, reverse transcription; *tk*, herpes simplex virus thymidine kinase gene.

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¶Deceased May 16, 1993.

and lysed by five cycles of freezing and thawing. CAT assays (27) used equal amounts of protein from each sample.

Transgenic Mice. A 4.2-kb fragment containing the 2342-bp *Eco*RI–*Hin*dIII fragment from the regulatory region of the rat ChAT gene followed by the *tk* minimal promoter, the CAT reporter gene, and polyadenylylation sequences was injected into pronuclei of fertilized mouse eggs to generate transgenic mice (28) (CBA × C57BL/6)F_1 mice were used as embryo donors, stud males, and pseudopregnant females. Mature C57BL/6 females were used for breeding. High molecular weight DNA from tail biopsies was prepared as described (28).

Sciatic Nerve Transection. Heterozygous transgenic mice, 3.5 weeks old, were deeply anesthetized with pentobarbital and both left and right sciatic nerves were transected at the mid-thigh level. Both the proximal and distal parts of ends of the axotomized nerves were reflected to minimize nerve regeneration. The operation did not prevent the mobility of the animals, which could readily access food and water supplies. Sham animals were similarly operated upon, but the sciatic nerve was not touched. Data were analyzed by Student's two-tailed *t* test for unpaired data.

Tissue Preparation, RNA Extraction, and Reverse Transcription (RT)–PCR Analysis. The age of embryos was determined by measuring crown–rump length. All tissues were immediately frozen on dry ice after dissection and stored at −70°C until analysis. The lumbar part of the cord was dissected and, while kept on dry ice, cut under the microscope into ventral and dorsal parts. For CAT assays, tissues were homogenized in 250 mM Tris (pH 7.5) with 20 strokes in a Dounce homogenizer. Samples were subsequently processed as described above for cultured cell lines. RNA was extracted (5) and RT–PCR was performed with 2 μg of total RNA according to manufacturer instructions (Perkin–Elmer).

RESULTS

Upstream Segment from the ChAT Gene Promoter Confers Cholinergic Specificity on a Noncholinergic Downstream Promoter *in Vitro*. Previous work identified a region of the ChAT gene promoter that directed gene expression to cholinergic cells *in vitro* (22). A 2342-bp 5′ flanking segment from this region, extending from an *Eco*RI site at −3865 to a *Hin*dIII site at −1523 (the transcriptional start site is defined herein as +1; P.L. and C.F.I., unpublished work), was subcloned in either orientation upstream of a *tk* minimal promoter driving expression of the bacterial CAT reporter gene. Both constructs were transfected into FR3T3 fibroblasts or into SN6 neuronal cholinergic cells, and transcriptional efficiency was measured by assaying CAT enzymatic activity. A construct with only the *tk* promoter and a promoterless construct were used as positive and negative controls, respectively. The *Eco*RI–*Hin*dIII fragment enhanced the activity of the *tk* promoter severalfold in SN6 cells as compared with the *tk* promoter alone (Fig. 1). This effect was observed regardless of the orientation of the *Eco*RI–*Hin*dIII fragment with respect of the downstream *tk* promoter. In contrast, transcription from the *tk* promoter in FR3T3 cells was greatly repressed by the *Eco*RI–*Hin*dIII fragment in either orientation (Fig. 1). This effect was due to the presence of specific silencer elements in this fragment (P.L. and C.F.I., unpublished work) and not to artifacts from vector sequences (29).

Regulatory Region in the ChAT Gene Promoter Directs Tissue-Specific Expression in Transgenic Mice. The heterologous construct containing the 2342-bp *Eco*RI–*Hin*dIII region upstream of the basal *tk* promoter and CAT reporter gene was microinjected into fertilized mouse eggs and transgenic lines were generated. Several other studies have demonstrated that the *tk* basal promoter is unable to direct cholinergic cell type-specific expression in transgenic mice when used alone or in combination with other regulatory sequences (30–32). Southern blot analysis identified 10 animals which had the transgene stably incorporated in their genome and that were able to transmit it to the offspring. Protein extracts from different brain regions and peripheral organs of F_1 animals were prepared and analyzed for CAT activity. Eight of the 10 lines were found to express the transgene. All 8 lines showed a similar regional distribution of CAT activity, as exemplified in Table 1 for the 3 lines displaying the highest levels of expression. However, expression levels did not correlate with transgene copy number (see legend to Table 1). High levels of CAT activity were found in the spinal cord of all expressing lines. In the brain of the 4-14 line, the levels and major sites of transgene expression correlated both qualitatively and quantitatively with areas previously shown to contain ChAT mRNA-expressing cells in the rat CNS (5), including pons, medulla, septum, olfactory bulb, and cortex (Fig. 2 *A* and *B*). Weak CAT activity in some of the brain regions represented true transgene expression as demonstrated by RT–PCR anal-

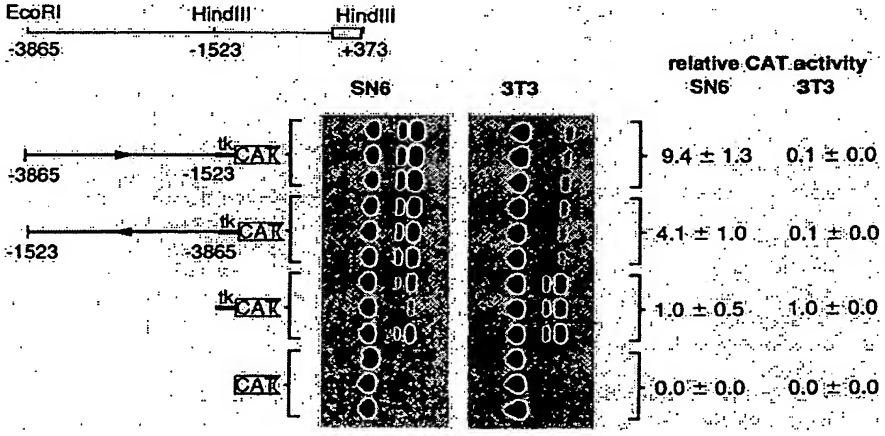

FIG. 1. Upstream 2342-bp *Eco*RI–*Hin*dIII ChAT gene segment confers cholinergic specificity on a heterologous *tk* promoter. Reporter gene expression was assayed as CAT activity in extracts of SN6 and FR3T3 cells transiently transfected in triplicate wells. CAT conversion values are given as mean ± SEM and are normalized to the value obtained with the *tk* promoter alone. A diagram of the upstream region of the ChAT gene is shown at the top; the 2342-bp *Eco*RI–*Hin*dIII fragment (shown as a thin solid bar in the lower diagrams) extends from position −3865 to −1523, transcription initiates at position +1, and the first exon of the ChAT gene is represented by an open box. Arrowheads in the 2342-bp enhancer segment indicate the relative orientations of this sequence with respect to the *tk* promoter (shown as a thick solid bar) and CAT reporter gene (open bar). The 156-bp sequence containing the *tk* promoter is not drawn to scale.

Table 1. Tissue distribution of CAT activity in three ChAT–CAT transgenic lines

Tissue	Relative CAT activity		
	3-13	4-14	4-17
Cortex	2.6	0.7	0.1
Cerebellum	4.7	1.2	0.0
Septum/striatum/thalamus/ hypothalamus	4.3	1.7	0.2
Pons/medulla/midbrain/ colliculi	9.5	5.1	2.4
Spinal cord	100	100	100
Thymus	2.6	1.5	0.6
Heart	0.0	0.0	0.1
Lung	0.1	0.0	0.1
Liver	0.8	0.1	0.1
Spleen	0.6	0.1	0.1
Pancreas	2.7	0.0	0.1
Duodenum	1.5	0.8	0.1
Kidney	0.1	0.1	0.0
Testis	0.3	0.1	0.0

Indicated tissues were dissected from 8-week-old mice from transgenic lines 3-13, 4-14, and 4-17. CAT assays used equal amounts of protein from homogenized extracts. Relative CAT activity in the spinal cord of each line was set to 100 (in this table and following figures, a relative CAT activity value of 100 in the adult spinal cord of lines 3-13, 4-14, and 4-17 corresponds to 1.5%, 2.2%, and 0.4% conversion of chloramphenicol per 10 μg of protein in 2 hr, respectively). Transgene copy number in lines 3-13, 4-14, and 4-17 was estimated at 1–2, 5–10, and 10–20, respectively, by Southern blot analysis (data not shown).

ysis (Fig. 2*C*). Transgene expression was also detected in areas known to receive cholinergic input, such as colliculi, thalamus, and hypothalamus (Fig. 2 *A–C*). No transgene expression was detected in the 4-14 line in hippocampus or striatum (Fig. 2 *A–C*). Overall, a similar pattern of transgene expression was observed in the brains of the 3 lines examined, with predominant expression in brainstem, basal forebrain, and cortex (Table 1). In 2 transgenic lines, CAT expression was detected in the cerebellum, a structure not previously shown to contain cholinergic neurons (Table 1). Various sites of weak peripheral expression were also observed in several transgenic lines, including thymus (5 of 8 lines), liver (5 of 8), kidney (3 of 8), duodenum (2 of 8), and pancreas and muscle (1 of 8) (Table 1, Fig. 2*C*, and data not shown). Interestingly, none of the 8 lines expressed the transgene in the testis, where germ cells have been shown to express ChAT mRNA at high levels (33).

To further establish the cholinergic origin of the transgene expression in the spinal cord, the lumbar enlargement of the spinal cord of transgenic animals was dissected and divided under the microscope into dorsal and ventral portions. For this experiment, the 3 lines showing highest expression of the transgene were chosen. Protein extracts were prepared and analyzed for CAT activity. In the 3 transgenic lines, 85–90% of the CAT activity was restricted to the ventral portion of the cord, where the cell bodies of cholinergic motor neurons are confined (Fig. 3). Together with the tissue distribution data, these results show that the 2342-bp regulatory region of the ChAT gene can direct cholinergic cell-specific expression from a noncholinergic promoter *in vivo*.

Transgene Developmental Regulation and Response to Nerve Injury Mimics Regulation of the Endogenous ChAT Gene. The developmental expression of the transgene was analyzed as CAT activity in spinal cords of animals from two different lines. The level of transgene expression increased slowly during embryonic development (Fig. 4). After birth, transgene expression increased more rapidly; the highest level was seen at 8 weeks of age, the latest time point examined (Fig. 4). Most importantly, transgene developmental regulation

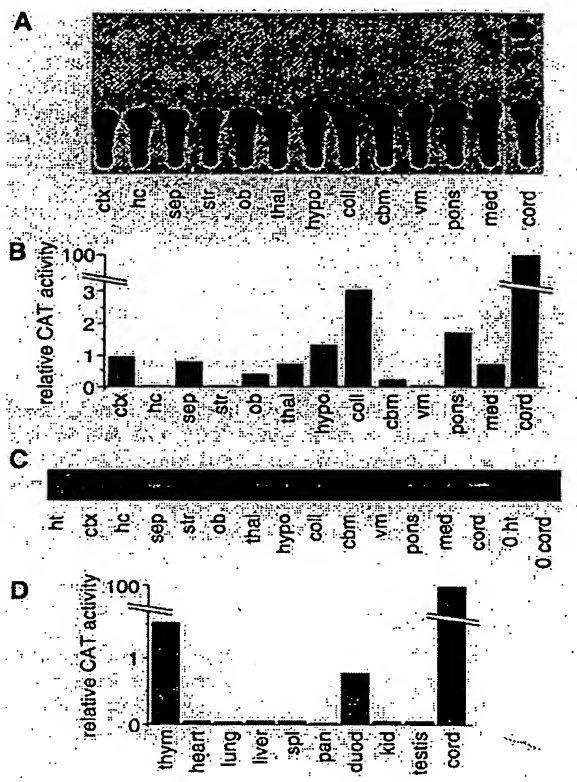

FIG. 2. Tissue-specific expression of CAT reporter gene in transgenic mice. (*A*) Autoradiogram of a CAT assay of tissue extracts from various brain regions from an 8-week-old transgenic mouse from line 4-14. ctx, Cortex; hc, hippocampus; sep, septum; str, striatum; ob, olfactory bulb; thal, thalamus; hypo, hypothalamus; coll, colliculi; cbm, cerebellum; vm, ventral midbrain; med, medulla; cord, spinal cord. (*B*) Quantitative distribution of transgene expression in the CNS of transgenic line 4-14. Relative CAT activity levels in spinal cord are defined here as 100. Abbreviations are as for *A*. (*C*) RT–PCR amplification of transgene mRNA in various brain regions from a transgenic mouse from line 4-14. Abbreviations are as in *A*, except ht, heart; 0 ht, nontransgenic heart; 0 cord, nontransgenic spinal cord. (*D*) Quantitative distribution of transgene expression in peripheral organs of transgenic line 4-14. Relative CAT activity levels in spinal cord (cord) are defined here as 100. thym, Thymus; spl, spleen; pan, pancreas; duod, duodenum; kid, kidney.

paralleled that of endogenous ChAT mRNA in rat spinal cord as analyzed by Northern blotting (Fig. 4).

Previous work showed a 2-fold increase in ChAT mRNA levels in spinal cord 7 days after a crush lesion of the sciatic nerve (5), probably reflecting part of a late regenerating response of motor neurons after injury. We therefore investigated the regulation of transgene expression in a paradigm of nerve damage. Bilateral sciatic nerve transection was performed in 3-week-old transgenic mice. CAT activity was assayed in protein extracts from the lumbar part of axotomized and sham-operated spinal cords 7 days after the operation. A significant 3- to 4-fold increase in CAT activity was observed in the axotomized spinal cord of transgenic animals compared with sham-operated controls (Fig. 5), suggesting that the promoter construct was also able to respond to nerve injury in a similar way as the endogenous gene.

DISCUSSION

We report that a 2342-bp regulatory region of the ChAT gene can confer cholinergic specificity on a downstream noncholinergic promoter in a cholinergic cell line and in transgenic

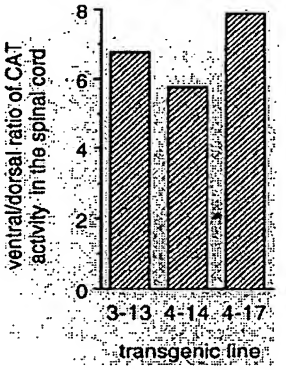

FIG. 3. Transgene expression is targeted to the ventral part of spinal cords from transgenic mice. The lumbar part of spinal cords of 8-week-old mice from the three indicated lines was dissected and divided into ventral and dorsal halves. The tissue was homogenized and CAT assays were performed. The ventral/dorsal ratio of CAT activity is shown for the indicated lines.

mice. This DNA segment enhanced the activity of a *tk* promoter in the cholinergic neuronal SN6 cell line but repressed expression from this promoter in the fibroblast cell line FR3T3. These effects were independent of the orientation of the 2342-bp fragment with respect to the *tk* promoter. These results suggest the presence of multiple regulatory elements that cooperate to control transcription initiation in cholinergic cells. In support of this hypothesis, we have recently identified a 21-bp silencer element related to an element previously shown to control neuron-specific expression of SCG10 and type II Na^+ channel genes in the proximal end of the 2342-bp fragment that is responsible for an important part of the silencer-like activity of this region in nonneuronal cell lines. In addition, other sequences localized to the same region appear to enhance the activity of a heterologous promoter in cholinergic cells. Sequence analysis of this region did not reveal any other sequence with similarity to previously described regulatory elements (P.L. and C.F.I., unpublished work). Thus, it appears that at least two kinds of regulatory elements in the ChAT gene are important for specific expression in cholinergic cells—namely, a neural-restrictive silencer element and a cholinergic-specific enhancer. The results of the present study further demonstrate that these elements are able to specifically direct gene expression to cholinergic regions of the CNS *in*

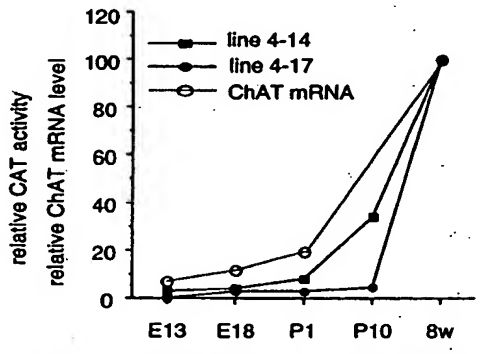

FIG. 4. Developmental regulation of transgene expression in the lumbar part of spinal cords from transgenic mice. Mice from transgenic lines 4-14 and 4-17 of the indicated ages were sacrificed and the lumbar part of the spinal cord was dissected, homogenized, and assayed for CAT activity. The developmental regulation of rat ChAT mRNA is shown for comparison (adapted from ref. 5). In the three curves, relative values at 8 weeks (8w) were set to 100, respectively. E13 and E18, embryonic days 13 and 18; P1 and P10, postnatal days 1 and 10.

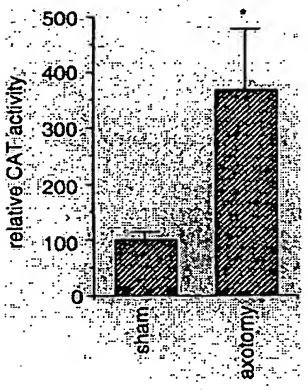

FIG. 5. Transgene expression in the spinal cord is increased after bilateral axotomy of the sciatic nerve. Sciatic nerves of 3-week-old mice from line 4-14 were bilaterally transected. Animals were sacrificed 7 days after the operation, and spinal cords were dissected, homogenized, and assayed for CAT activity. Bars show mean and SEM (*, $P < 0.025$; $n = 4$).

vivo. In all lines examined, the highest level of transgene expression was found in the spinal cord, followed by the pons and medulla (10- to 40-fold lower than spinal cord), nuclei of the basal forebrain, and cortex. The relative levels of transgene expression in CNS regions were in good agreement with the distribution of ChAT mRNA in the rat CNS (5). In contrast to previous reports in other rodents, a recent immunocytochemical study found no cholinergic cell bodies in mouse cerebral cortex (34). Basal forebrain cholinergic neurons, therefore, appear to provide the main cholinergic input to cortical cells in mice. CAT activity in cortex and in some other brain regions of ChAT–CAT transgenic mice, such as thalamus, hypothalamus, and colliculi, could therefore be the result of anterograde transport of the reporter gene product to terminals of cholinergic afferents, such as those from the basal forebrain and pedunculopontine tegmental nucleus. No transgene expression was detected in the striatum, which is known to contain sparse cholinergic interneurons. In the lumbar enlargement of the spinal cord, 85–90% of the transgene expression was targeted to the ventral part of the cord, where cholinergic α-motor neurons are located. Thus, the pattern of expression of the transgene agrees well both qualitatively and quantitatively with that reported for ChAT mRNA in various regions of the rat CNS.

The cholinergic phenotype of spinal cord motor neurons develops gradually during embryonic life. The level of ChAT mRNA increases slowly during embryonic development but more rapidly after birth, reaching maximal levels in the adult spinal cord (5). The transgene developmental regulation in the spinal cord mimicked that of the endogenous gene, indicating that control of tissue-specific expression and developmental regulation in the ChAT gene may be mediated by closely located, or even identical, elements and may therefore share common molecular mechanisms. Furthermore, the fact the transgene responded to nerve injury in a similar way as the endogenous gene suggests that these elements may also play an important role in the control of the cholinergic phenotype in injured neurons.

ChAT mRNA and protein have previously been detected in rat and human male germ cells (33), although the role of acetylcholine in sperm cell maturation and function remains to be established. None of the transgenic lines generated showed detectable levels of transgene expression in the testis. We have recently found that transcription initiates from the promoter region analyzed here in spinal cord and brain but not in testis (P.L. and C.F.I., unpublished work), suggesting the existence of alternative sites for the initiation of ChAT gene transcrip-

tion in testis. Previous reports indicated the presence of two additional promoters in the ChAT gene located downstream of the sequences studied here, from which transcription appears to also initiate in the spinal cord (20, 21). The individual contribution of the different promoters to ChAT gene expression in different tissues is unknown. Our results suggest that transcription from the ChAT gene in the testis may be differentially controlled from one of these alternative downstream promoters or from as yet uncharacterized regions of the gene. The regulatory region analyzed in this study may be useful to specifically target gene products to cholinergic neurons. This approach could prove useful for the generation of animal models for human diseases where degeneration of cholinergic neurons occurs, such as Alzheimer disease or amyotrophic lateral sclerosis.

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Original article

Expression of a human insulin transgene in cholinergic neurons of the mouse medial habenula

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Summary – We explored the possibility that an insulin gene deleted in its 5'-flanking region is expressed in adult mouse brain. We used three independent lines of mice carrying a human insulin transgene which included the insulin gene transcription unit flanked by 168 base pairs upstream and 5.5 kb downstream. Using a reverse transcription-polymerase chain reaction assay, human insulin mRNAs were detected in whole brain extracts. In all three lines, human insulin mRNAs were localized by *in situ* hybridization in a single cerebral site, the medial habenula. With a monoclonal antibody specific for human C-peptide and human proinsulin, labelling was restricted to a subset of habenular cholinergic neurons, with rare immunostained fibers. No labelling was observed in the projection fibers of the retroflexus fasciculus or in their axon terminals in the interpeduncular nucleus. Electron microscope studies suggested that the processing of the human peptide involved the endoplasmic reticulum and Golgi apparatus, but there were no secretory granules in the transgene expressing cells. These findings demonstrate that the human insulin transgene tested here includes a habenula specific promoter which could be useful for physiological and molecular studies on the habenula.

medial habenula / cholinergic neurons / transgenic mouse / insulin / immunocytochemistry

Introduction

The effects of 5'-flanking sequence deletions on the expression of the human insulin gene in transgenic mice have already been reported [13]. The gene constructs tested included the transcription unit of the human insulin gene (1430 bp) and 5.5 kb of its 3'-flanking region. These constructs differed in the length of their 5'-flanking sequences, which had 353 (Δ-353), 258 (Δ-258), 168 (Δ-168) or 58 (Δ-58) nucleotides upstream of the transcription start site, *ie* the nucleotide +1.

Northern blot analysis of the total RNA extracted from various organs, including the pancreas and brain, showed that first, the human insulin gene was expressed in the pancreas only, except for the Δ-58 construct, which was not expressed in any organ; second, the shorter the gene construct, the lower the level of human insulin expression. Therefore, the expression level of the human insulin gene in pancreas transfected with the Δ-168 construct was very weak. As this construct contains a few regulatory elements in its 5'-flanking region, we wondered whether it allows extrapancreatic expression of human insulin, at levels which would be below the sensitivity threshold of Northern blot analysis.

We were interested in brain expression for two reasons: first, insulin genes are expressed in the brain cells of non-transgenic rat and mouse at very low levels. Although this expression is hardly detectable by *in situ* hybridization [35] and is not observed using Northern blot analysis [14] or immunocytochemistry [1], it was recently shown in rat and mouse brain by RT-PCR ([11] and Bucchini, unpublished observations). Second, the brain cells which might express endogenous insulin genes at low levels, may also be able to express human insulin. Several copies of a transgene are usually integrated in a same chromosomal site [18]. If the brain cells expressing mouse insulin can express several copies of the transgene at the same time, these cells should be detectable using *in situ* hybridization and immunocytochemistry for human insulin-related molecules. Thus, the study of the cerebral expression of the Δ-168 construct may help to localize brain cells expressing mouse insulin genes.

Preliminary cytochemical studies have shown that mice from three independent Δ-168 transgenic lines expressed the human insulin gene in a very limited region of the brain, the medial habenula [12].

In the present study, we undertook a more thorough study of the expression of the human transgene in the brain of Δ-168 mice. Several antibodies directed against human proinsulin, mouse C-peptides, human C-peptide or insulin were used to check the specificity of immunolabelling, to investigate a possible human proinsulin transformation into insulin and to show any expression of mouse insulin genes in cells expressing the human transgene. As the medial habenula was reported to harbour mainly cholinergic or substance P-containing neurons [8, 17, 30] and astrocytes [36], we attempted to identify the cell type which expresses the human insulin gene, using antibodies directed against choline acetyltransferase (ChAT), substance P (SP) or glial fibrillary acidic protein (GFAP).

Abbreviations: RT-PCR, retrotranscription-polymerase chain reaction; ISH, *in situ* hybridization; PBS, phosphate buffered saline; DAB, diaminobenzidine; BDHC, benzidine dihydrochloride; HPI, human proinsulin; HCP, human C-peptide; GFAP, glial fibrillary acidic protein; ChAT, choline acetyltransferase; SP, substance P.

Materials and methods

Transgenic mice

The transgenic mice and their production have been described [13]. They carried the human insulin gene (1430 base pairs), 168 base pairs upstream from the transcription start site, and 5.5 kb of its 3'-flanking region. Three independent lines of transgenic mice (in all, 40 adult mice) were used. Mice were heterozygous for the human transgene and were identified by a PCR performed on tail DNA. Transgene expression in brain was tested by RT-PCR, *in situ* hybridization (ISH) and immunocytochemistry at the light and electron microscope levels.

Retrotranscription and polymerase chain reaction (RT-PCR)

RNA was prepared from fresh brains of adult mice by the guanidine thiocyanate procedure and then centrifuged through CsCl as described previously [7]. Sixty micrograms of total RNA was obtained from each brain and treated with DNase, and 2.5 μg was then submitted to RT-PCR as described elsewhere [10]. We used a pair of oligonucleotide primers, 5' (5'-ATCACTGTCCTTCTGCC-3') and 3' (5'-GACCACCTCCGTCGGACGTC-3'), which were homologous to the sequence of human insulin gene fragments (respectively at the beginning of the second exon, 2407–2424, and in the C-peptide coding sequence 3424–3443 [2]). After RT and PCR, each cycle consisted of 1 min at 95°C, 1.5 min at 55°C, and 1.5 min at 72°C, a 252 bp DNA fragment was obtained and purified by agarose gel electrophoresis, transferred onto Gene Screen Plus membrane and analyzed by Southern hybridization. A single oligonucleotide probe (5'-ACAATGCCACGCTTCTG-3'), 5'-end labelled with [γ-^{32}P] ATP to a specific activity of 2×10^9 cpm/μg was used. The membranes were hybridized overnight at 42°C, washed (final wash in 0.1 × standard saline citrate (SSC)/1% SDS, at 42°C), and exposed to Amersham Hyperfilm-MP.

Hypoxanthine-guanine phosphoribosyltransferase (hprt) mRNA in the samples was also amplified as a control. The primers were 5' (5'-GATGATGAACCAGGTTATGA-3') and 3' (5'-ATGTCCCCCGTTGACTGA-3'), and the probe was 5'-TTATCAGACTGAAGAGCTAC-3'. Because there was no interference between the amplification of human proinsulin and of hprt cDNAs, both amplifications were done in the same tube. In each RT-PCR series, a control tube was included containing all the reaction elements except RNA.

Stereotaxic injections

To visualize neurons containing choline acetyltransferase and substance P in the medial habenula, mice were anesthetized IP with 80 mg/kg nesdonal, fixed in a stereotaxic apparatus and injected with 1 μl of a 2 mg/100 μl colchicine water solution (Boehringer Mannheim) in the right lateral ventricle. The stereotaxic coordinates from the bregma were those indicated by Lehmann [21], *ie*: anteroposterior plane, 0.00; lateral to the midline, 1.10 and 2.80 mm below the surface of the skull. Operated animals were left to recover for 36 h and treated as described below.

Tissue preparation

Animals were deeply anesthetized with 6 mg/kg sodium pentobarbital (Sanofi, 33501 Libourne, France) and perfused at room temperature *via* an intra-aortic cannula with: 0.1 M phosphate buffered saline (PBS) (pH 7.4) containing 0.1% sodium nitrite, and then with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed, postfixed for 1 h in the same fixative and cryoprotected in 12% sucrose PBS at 4°C overnight. Twelve-micrometer thick sections were cut with a cryostat and collected in sterile PBS.

Tissue processed for electron microscopy was fixed with 0.1% glutaraldehyde-4% paraformaldehyde PB and cut into 50-μm thick slices using a vibratome.

In situ hybridization

In situ hybridization experiments were carried out using a synthetic oligodeoxynucleotidic probe (20-mer, 5'-CTGCAGGCTGCCTGCACCAG-3'), complementary to the sequence encoding for aminoacids 72–78 of human preproinsulin [2]. The probe was labelled for 1 h at 37°C by tailing with [α-^{35}S] dATP (10 μM, 37 Tbq/mmol, Amersham, UK) using 20 units of calf thymus terminal deoxynucleotidyltransferase (Boehringer Mannheim, Germany). The resulting probe specific radioactivity was about 220 Tbq/mmol.

After three rinses in PBS, sections were incubated in the following solutions: 1) prehybridization buffer (4 × SSC, 1 × SSC: 0.15 M NaCl, 0.015 M tri-sodium citrate) and Denhardt's solution comprising 0.02% Ficoll (Sigma, St Louis, MO 63178, USA), 0.02% polyvinylpyrrolidone (Sigma) and 0.02% bovine serum albumin (Sigma)), for 1 h at 37°C; 2) hybridization buffer (4 × SSC, Denhardt's solution, 50% deionized formamide, 10 mM dithiothreitol, 0.04% diethyl pyrocarbonate (Sigma), 1 nM labelled probe), overnight at 37°C; 3) washes: 2 ×, 1 ×, 0.5 × and 0.1 × SSC, two 30-min rinses at 37°C for each solution. Sections were mounted on slides coated with gelatin chrome-alum, air dried, and either exposed to an autoradiographic film (Amersham β-max Hyperfilm) or dipped into a nuclear emulsion (Ilford K5). Films and emulsion-treated slides were respectively developed 5 and 10 days later.

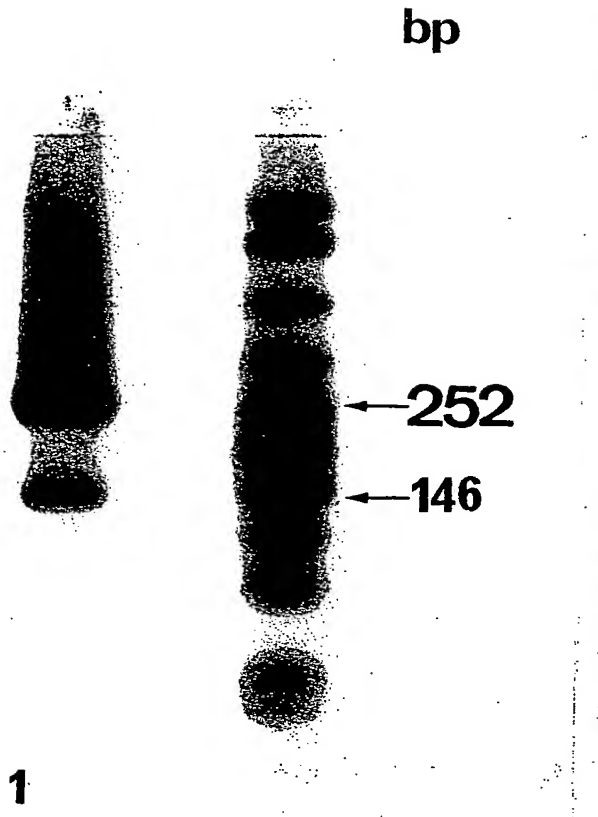
To check labelling specificity, some sections were submitted to RNase pretreatment at 37°C for 45 min with RNase I 'A' from bovine pancreas (Pharmacia, Sweden) or incubated in hybridization buffer with an excess of unlabelled probe (10 nM) and processed as above.

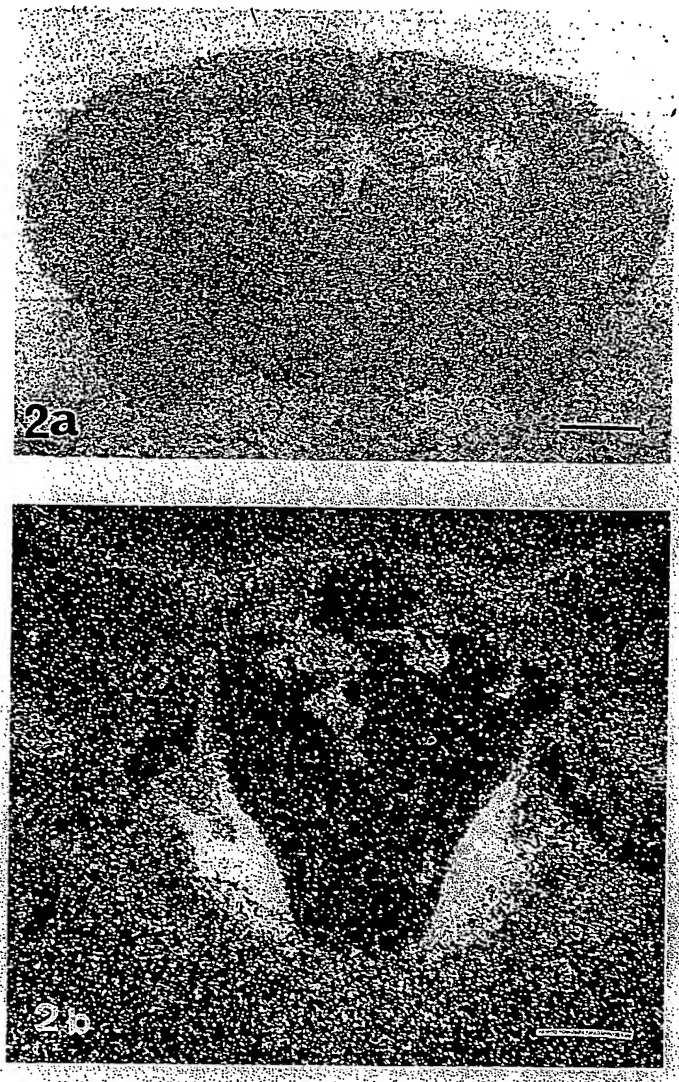
Immunocytochemical studies

Mouse and human insulins and C-peptides, as well as some cytochemical markers, were explored using a previously described immunoenzymatic technique [16], with the avidin-biotin-peroxidase complex (ABC). Briefly, antigens were stained overnight at 4°C on free-floating sections using the following antibodies: mouse anti-human proinsulin (HPI) monoclonal antibody (1/1000; HPI-005; Novo Nordisk, Copenhagen, Denmark); rat anti-human C-peptide (HCP) monoclonal antibody (1/4000; GN-ID4; [25]); guinea pig anti-bovine insulin polyclonal antibody (1/4000; Novo Nordisk); rabbit anti-mouse C-peptide-I (1/2000; no 657) and anti-mouse C-peptide-II (1/500; no 660) polyclonal antibodies [4]; rabbit anti-glial fibrillary acidic protein (GFAP) antiserum (1/1000; Z334; Dako A/S, Denmark); rabbit anti-substance P (SP) antiserum (1/1000; [3; 19]) and goat anti-rat/mouse choline acetyltransferase (ChAT) antiserum (1/1000; AB144; Chemicon, Temecula, CA 92590, USA).

Sections were incubated for 1 h at room temperature with the corresponding secondary biotinylated antibodies directed against guinea pig, rabbit, rat or goat IgG (1/250; Vector Laboratories, Burlingame, CA 94010, USA), and then for 1 h at room temperature with the avidin-biotin-peroxidase complex (1/25; Elite ABC kit, Vector Laboratories). Bound peroxidase activity was visualized on sections using 3,3'-diaminobenzidine (DAB, Sigma) as chromogen [16]. Between each step, sections were rinsed three times with PBS. ChAT or SP immunoreactivities were detected on sections of colchicine-treated brains.

Double-label detection was done on the same brain sections, using a combination of two immunoperoxidase methods [20, 22]. Sections were incubated overnight at 4°C with two primary antibodies obtained in different species. Section-bound primary antibodies were successively detected as described above, using DAB, which yields a diffuse brown deposit, as chromogen for the first revelation and benzidine dihydrochloride (BDHC, Sigma) for the second. When the pH of both the incubation medium and the post-incubation rinses was less than 7.0, the BDHC deposit was large, crystalline, and deep blue, but when the pH rose above 7.0, it became diffuse and brown, similar to DAB. For this reason, a PB6 0.01 M phosphate sodium buffer (pH 6.6), was used to reveal peroxidase activity with BDHC. Previous authors reported that the peroxidase complex linked to





Figs 1, 2. 1. Detection of human insulin gene messenger RNAs in transgenic mouse brain. Radioautogram of RT-PCR products after separation by agarose gel electrophoresis and hybridization with human insulin and hprt radiolabelled probes. Left lane, the human amplified fragment of 252 bp and the hprt fragment of 146 bp; right lane, *Hpa*II digest of pBR 322. **2.** Radioautographic film (a, scale bar, 1 mm) and nuclear emulsion coated slides in dark field view (b, scale bar, 100 μm) after *in situ* hybridization with a human insulin ^{35}S-labelled oligonucleotide probe. Labelling is visible in the ventral part of the medial habenula on the radioautographic film and emulsion-coated slides where large aggregated grain clusters can be seen. Hippocampus and choroid plexus labellings were shown to be unspecific.

the first antigen did not catalyse the reaction product during the subsequent BDHC incubation. The DAB reaction product was previously suggested to mask the access of the second chromogen [20, 22]. To check the specificity of the double detection in our experiments, two control experiments were carried out: 1) a single detection test with anti-HCP antibody, in which antibody-linked peroxidase activity was revealed, first with DAB and then with BDHC after five rinses in PB6; and 2) after DAB revelation, some sections were incubated with ABC complex before the second peroxidase revelation with BDHC.

For electron microscopy examination, sections were incubated with the HCP antibody and their peroxidase activity was revealed with DAB. They were postfixed for 1 h in 2% osmium tetroxide in PB, dehydrated in graded alcohols, embedded in Epon resin (Polyscience, Warrington, PA 18976, USA) and cut into ultrathin sections for observation with a Jeol 100 CX electron microscope.

In the habenula, *in situ* hybridization and immunocytochemical detection were done on successive sections that were counterstained with cresyl-violet and mounted in Permount (Fisher Chemical, Fair Lawn, NJ 07410, USA). A more extensive immunocytochemical brain examination was carried out using the HCP antibody on sections spaced 100 μm apart, collected from olfactory bulbs to the caudal part of medulla.

Results

Detection of messenger RNAs (figs 1, 2)

Retrotranscription and polymerase chain reaction (fig 1)

After hybridization with an insulin probe, RNAs prepared from adult mouse brains and submitted to RT-PCR, displayed an amplified fragment of 252 bp. This fragment had the expected length, due to the relative positions of the

primers on the human insulin cDNA. The hprt probe detected an amplified fragment, which also displayed the expected length (146 bp). Neither fragment was found in control experiments without RNA in the reaction tube (data not shown).

In situ hybridization (fig 2)

In the three independent lines tested, histologic radioautography of coronal sections hybridized with the human insulin probe showed a completely uniform film darkening over the whole section, with slightly deeper darkening over the hippocampus, choroid plexus and medial habenula (fig 2a). After pretreatment with ribonuclease or incubation with an excess of unlabelled probe, the medial habenula labelling was the only one to disappear, suggesting that it was due to specific hybridization with a ribonucleic acid. Cellular radioautography of emulsion-coated sections resulted in uniform silver grain density over the whole section, with grain accumulation over the choroid plexus, dentate gyrus and ventral part of the medial habenula, where big grain clusters aggregated (fig 2b). The habenular labelling was the only one to disappear in control experiments with RNase or excess of unlabelled probe. No such labelling was observed elsewhere when the whole brain was explored.

Immunocytochemical detection of the insulin gene-related peptides (figs 3, 4)

In the three independent lines tested, incubation with a single antibody directed against human proinsulin (fig 3a), human C-peptide (fig 3b) or bovine insulin (fig 3c), revealed a red-brown precipitate resulting from a peroxidase reaction, in a cell subset of the medial habenula on successive sections. Whatever the antibody used, the cells stained red-brown were mainly located in the ventro-medial part of the nucleus, under the unstained ependyma. Most of them were organized in parallel arrays although a few were scattered more laterally. Nevertheless, comparison of the HPI- and HCP-immunoreactive cell populations on successive sections showed that the population of HPI-immunoreactive cells was smaller (fig 3a,b). Labelled cells were rather round and exhibited a few short labelled processes; some rather thin long elements stained brown resembled cell processes (see inserts in fig 3a–c). Extensive brain examination, with HCP antibody on sections spaced 100 μm apart did not reveal labelling in medial habenula projection fibers, the interpeduncular nucleus or any other medial habenula efferent structures.

When brain sections were incubated with anti-mouse C-peptide I antibody (fig 4, left panel) or mouse C-peptide II antibody (fig 4, right panel), the very slight and uniform staining observed was identical to the background staining obtained with rat preimmune serum (data not shown).

Colchicine treatment reduced medial habenula width due to habenular cell flattening, but no changes were observed in the number or location of HCP-immunoreactive cells.

Double immunocytochemical detection (figs 5, 6)

In each double detection, the first peptide cited was revealed using DAB (red-brown precipitate signal) and the second one with BDHC (dark-blue grain signal). In all the double detection experiments, on the three independent lines tested, red-brown background staining was stronger than in the single detection tests, whereas the dark blue grain background was weak and uniform.

GFAP and HCP detection (fig 5)

Red-brown cells and filamentous elements were observed. They formed a dense network mainly under the unlabelled ependyma. Round spaces not labelled with red-brown DAB precipitate were clearly delimited. Some of them were covered by dense dark blue grain clusters, sometimes with a clearer central area. No grain clusters were observed over cells stained red-brown. This indicates that the HCP-immunoreactive cells labelled with dark blue grains were not the same as the GFAP-immunoreactive cells labelled red-brown.

HCP and SP detection (fig 6a)

Big clusters of dark blue grains were observed in the dorsal part of the nucleus, whereas many red-brown cells were seen in the ventral part. Between these areas, red-brown cells were observed near grain clusters but there was never any overlapping. This suggests that the HCP-immunoreactive cells stained red-brown do not contain SP.

ChAT and HCP detection (fig 6b)

Cells stained brown were observed mainly in the ventral part of the medial habenula. A few of these cells were covered with dark blue grains, and all the grain clusters covered brown cells. These observations suggest that HCP-immunoreactive cells form a subset of the ChAT immunoreactive neurons.

In control experiments, a single primary antibody was used (GN-ID4) and the two chromogens DAB (red brown) and BDHC (dark-blue) were successively applied to the section-linked peroxidase activity. Only red-brown cells were observed, which indicates that the second chromogen only accumulates when two primary antibodies are bound in the same cell (data not shown).

Human C-peptide detection at ultrastructural level (fig 7)

After incubation with the HCP antibody and peroxidase revelation with DAB, discrete small electron-dense aggregates were observed in habenular cells, mainly located close to the unlabelled ependyma. No such aggregates were observed after incubation with preimmune serum or in the absence of DAB oxidation. The labelled cells displayed an indented nucleus. No synaptic contacts on the cell body membrane were evidenced. Labelled cells did not display secretory granules. DAB precipitates were localized on the inner surface of the outer membrane of the nuclear envelope, in the restricted part of the rough endoplasmic reticulum, and at the extremity of some Golgi saccules. Some precipitates were observed in the cytoplasm, near the plasma membrane.

Discussion

In this study, we investigated the expression of a human insulin transgene deleted in its 5'-flanking region in adult mouse brain, and we report the following significant findings: 1) human insulin mRNAs were present in total RNA brain extract; 2) human insulin mRNAs were only found in the ventral part of the medial habenula and nowhere else in the brain; 3) human proinsulin immunoreactive material was only detected in a cell subset of the ventral part of the medial habenula; 4) human C-peptide and choline acetyltransferase immunoreactivities were colocalized in habenular cells; 5) human C-peptide immunoreactive material was present in the endoplasmic reticulum and Golgi apparatus in

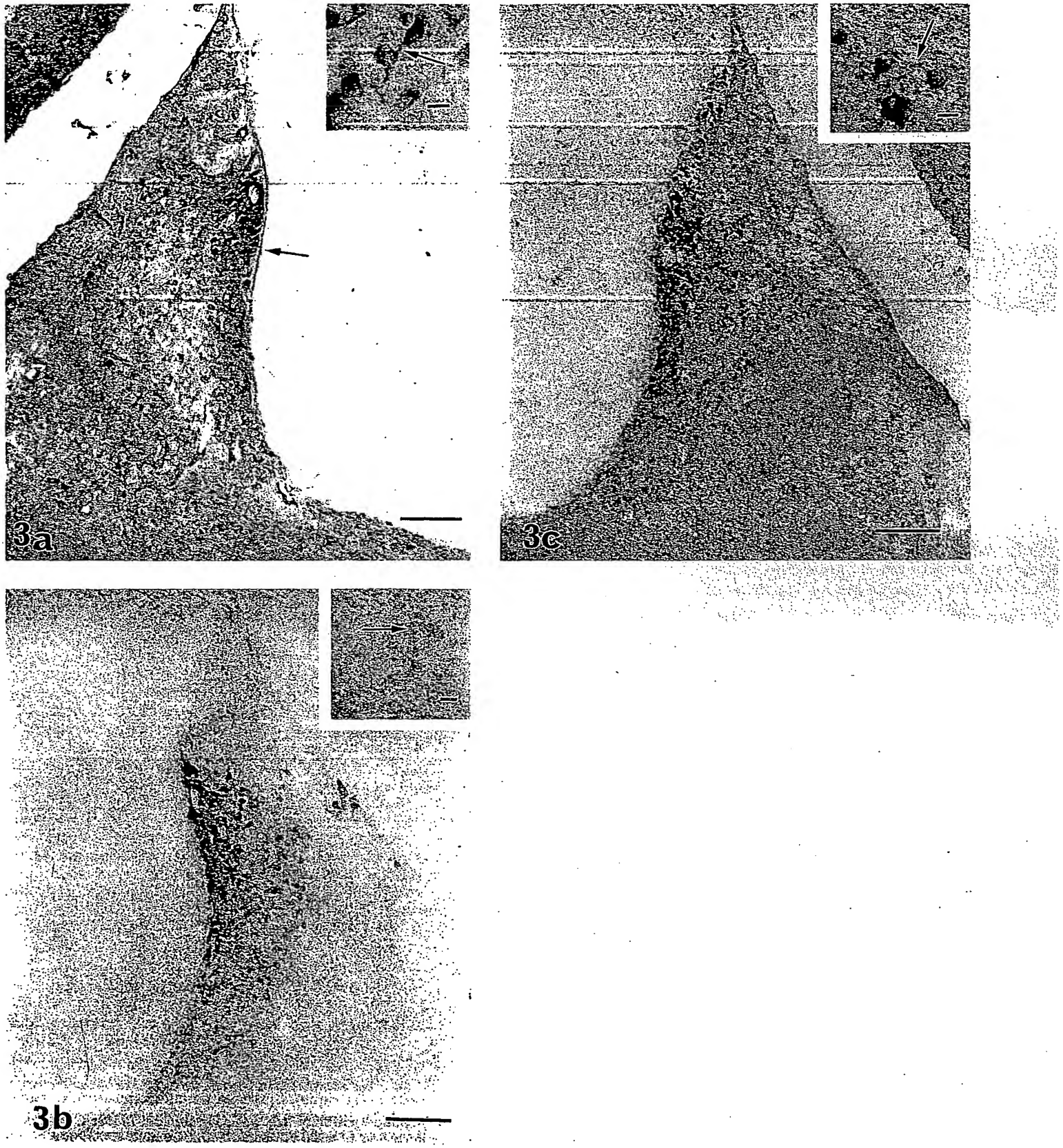
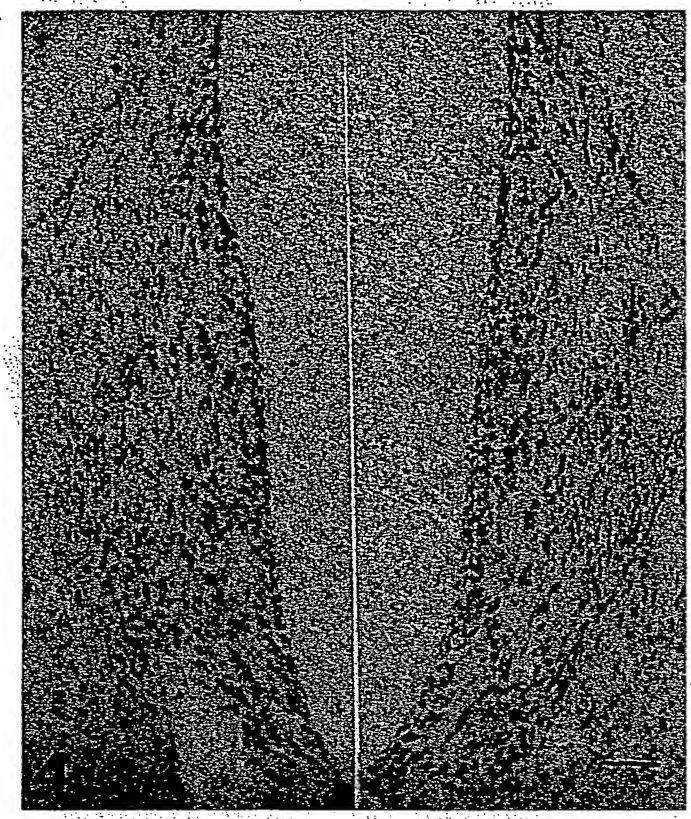


Fig 3. Immunocytochemical labelling of the medial habenula using antibodies to human proinsulin (a), human C-peptide (b) and bovine (c) insulin (scale bar, 30 μm). Labelled cells are mainly located under the unlabelled ependyma (arrow) and display labelled processes (inserts, scale bar, 10 μm).



Figs 4–6. 4. Absence of habenular immunolabelling using the anti-mouse C-peptide I antibody (left panel) or the anti-mouse C-peptide II antibody (right panel) (scale bar, 100 μm). 5. Double immunoperoxidase detection of glial fibrillary acidic protein (DAB red-brown labelling) and human C-peptide (BDHC dark blue grains) in the habenula. No colocalized signals are visible (scale bar, 20 μm). 6. Double immunochemical detection of neurocytochemical markers and human C-peptide. **a.** Substance P-immunoreactive neurons are labelled with dark blue grains and differ from human C-peptide immunopositive cells, which are stained in brown (arrow). **b.** Among brown single stained cells, immunoreactive for choline acetyltransferase (single arrow), some cells are also reactive for the human C-peptide and doubly labelled with brown DAB precipitate and dark-blue grains (double arrow). Scale bar: a, 50 μm; b, 20 μm.

habenular cells devoid of secretory granules; and 6) no material reactive to antibodies directed against mouse C-peptides I or II was detected in the brain and more precisely in the medial habenula of the transgenic mice examined.

The oligonucleotides used for RT-PCR were specific for human insulin cDNA because of their weak homology with respect to mouse insulins and IGFs cDNA sequences. The probe used for *in situ* hybridization (ISH) only exhibited seven and 14 nucleotides out of 20 which were homologous respectively to the corresponding mouse insulin I and II sequences. It was previously shown that our ISH experimental conditions did not allow a 20-mer probe to hybridize with mRNA sequences displaying such a weak homology [33]. The use of ^{35}S as radioisotope for ISH did not give a cellular resolution, due to the small size of the habenular neurons and their dense organization. Nevertheless, the radioautographic signal was clearly located in the same part of the medial habenula as the part exhibiting human insulin *(hins)* gene-related immunolabelling, which corroborates the specificity of the ISH results.

The specificity of our immunocytochemical data is also supported by the similar distributions of the labelled cells shown on successive sections using antibodies to human proinsulin, human C-peptide and bovine insulin. The human C-peptide antibody does not recognize the mouse C-peptides I and II, whereas the bovine insulin and human proinsulin antibodies recognize both human and mouse (pro$^-$) insulins ([25] and data not shown). Consequently, the immunolabelling obtained with the human C-peptide antibody clearly shows the presence of human molecules in the medial habenula.

Despite the structural homology of the human and mouse insulin genes, the different techniques used here did not reveal the expression of mouse insulin genes in the habenula and our results are consistent with the specific expression of the human insulin gene in this tissue.

The hypothesis that the (–168/–58) fragment is involved in the activity of the transgene in habenular cells is supported by the fact that the data reported here were obtained from three independent transgenic mouse lines. Chromosomal integration of the transgene appears to be random, and cytogenetic analysis of other transgenic mouse lines has shown that an 11 kb DNA fragment including the *hins* gene was integrated in different chromosomes from one line to another [27]. Therefore, we can assume that in all three lines, the human insulin transgene was randomly integrated into chromosomal DNA, and that its exclusive medial habenula expression is not due to the genetic environment of the insertion site, but indeed to the transgene nucleotide sequence. Thus, a human insulin transgene maintaining a few regulatory elements in its (–168/+1) 5'-flanking region [5, 6] can be expressed in adult brain.

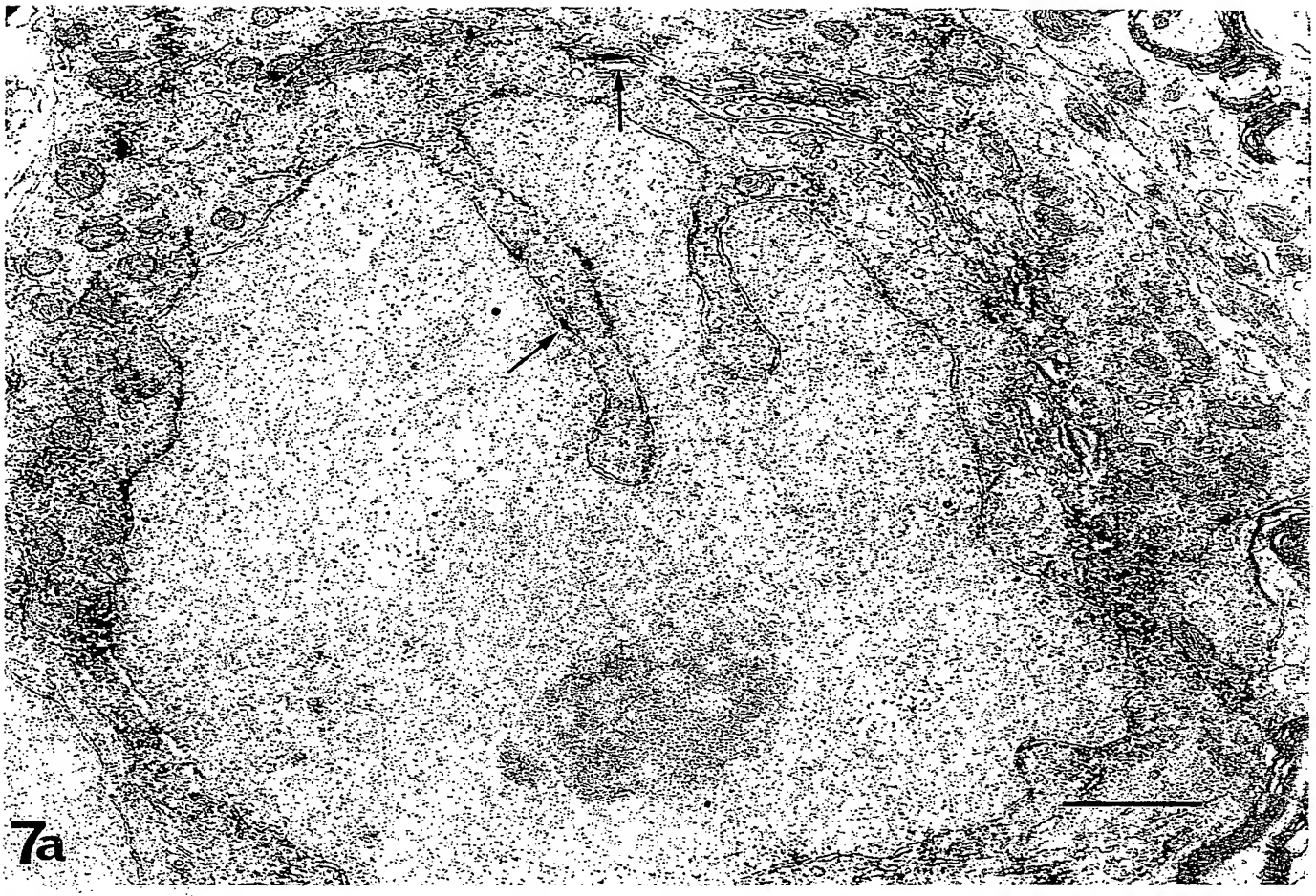
In fact, the 168 nucleotides located upstream from the transcription start site include the *hins* gene promoter (–58/+1) and the (–168/–58) fragment. This fragment is essential, because its deletion completely abolishes *hins* transgene expression [13]. The expression of the Δ-168 human insulin transgene in habenular cells is not understood. We cannot exclude that the specific expression observed in the medial habenula is a fortuitous event, which displays no link with the expression of the mouse insulin genes. For instance, neuronal specific expression of several fusion transgenes (*eg* fusion of the promoter of the metallothionein gene and growth hormone [24, 31] or somatostatin [23] coding sequence with its 3'-flank) was described in brain areas that do not normally express the corresponding endogenous genes. The unexpected expression of these fusion transgenes could be due to a particular combination of regulatory sequences from two genes with different tissue specificity. However, the Δ-168 transgene is not a fusion gene and other hypotheses have to be viewed. It might be that the (–168/–58) sequence harbours elements allowing the expression of the gene in habenular cells whereas negative elements are present in the upstream sequence. Alternatively, deletion of the DNA fragment upstream of –168 might 'open up' the region, allowing transcription factors present in habenular cells to reach the promoter region of the transgene. Another possibility is that endogenous insulin genes are very weakly expressed in habenular cells and that neither the 20-mer probe used for *in situ* hybridization nor the specific antibodies used were able to detect them.

Whatever the reason for the expression of the Δ-168 *hins* transgene, it was important to determine what cell type is marked by this new molecular probe.

The cell type to which habenular proinsulin synthetizing cells belong was investigated on sections of brains injected with colchicine in order to visualize somata containing ChAT and substance P. Although intracerebroventricular injections of colchicine were found to affect gene expression [9], they did not lead here to any observable changes in brain HCP immunoreactivity. We can therefore assume that the HCP-immunoreactive cell population shown in the double immunocytochemical detection experiments was the same as that observed in mice not treated with colchicine.

No colocalization of labelling was observed using the anti-GFAP and anti-HCP antibodies, which suggests that the cells expressing proinsulin were not astrocytes (fig 5). Since the rat medial habenula was reported to harbour mainly cholinergic or substance P-containing neurons [8, 17, 30], we checked the distribution of these neuromediators in the transgenic mice and found that it was identical to the distribution in rat and non-transgenic mice, *ie* substance P-containing neurons were located dorsally, and cholinergic neurons, ventrally (data not shown). As HCP-immunoreactive cells were labelled ventrally, and substance P-containing neurons, dorsally, they are clearly different (fig 6a). ChAT immunoreactivity was always observed in HCP-IR cells, but on the other hand numerous cells exhibited ChAT labelling only (fig 6b). Therefore, cells expressing proinsulin probably constitute a subset of cholinergic habenular neurons.

We observed more HCP-immunoreactive cells on GFAP/HCP double detection slices (fig 5) than on ChAT/HCP double detection slices (fig 6b). As double staining often reduces the intensity of cytochemical labellings, DAB deposit of the first peroxidase revelation might have masked access to the antibodies directed against HCP which were mixed with anti-ChAT antibodies at the beginning of the experiment. Furthermore, single immunodetection demonstrated that ChAT immunoreactivity was weaker in habenular neurons than in other cholinergic cells (*eg* substantia nigra), even in colchicine injected brains. For this reason, we had to prolong the incubation with DAB in order to reveal habenular ChAT labelling. These points might account for the loss of sensitivity we observed with the BDHC revelation. There were probably no cells expressing HCP without ChAT, since such cells, which should be clearly labelled with the BDHC reaction product and background DAB deposit, could not be seen in the habenular sections shown (fig 6b). Because of the loss of sensitivity of the BDHC reaction, we cannot exclude the possibility that in cells which might express HCP at very low levels, this reaction was prevented by the DAB background deposit. Habenular neurons also express neurokinin B [26], but the low level of transgene expression in these cells might be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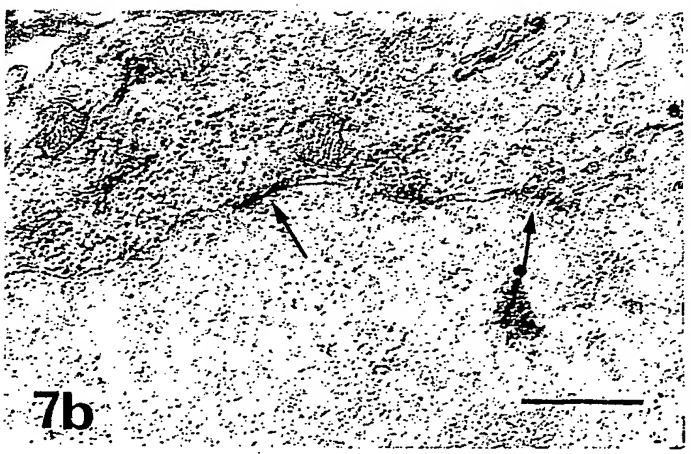
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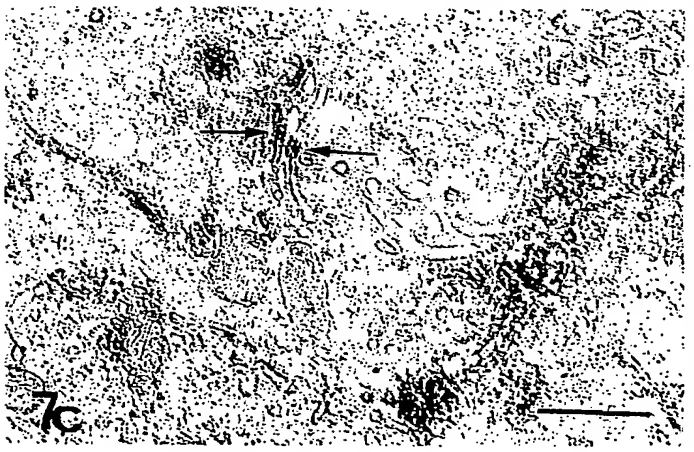
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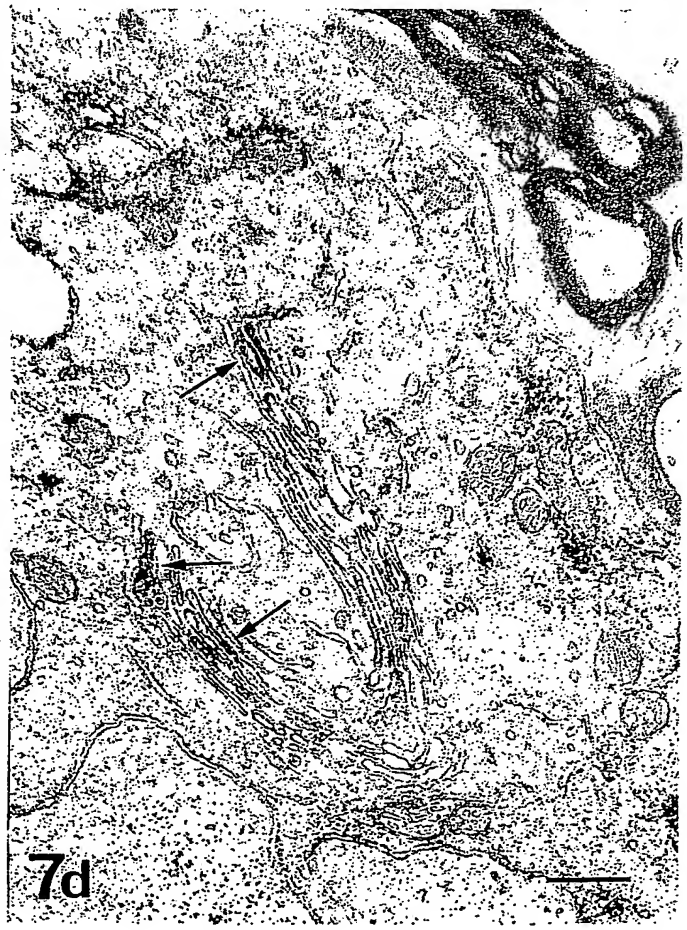
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incompatible with most of the protocols for double immunocytochemical detection.

We carried out ultrastructural studies to define the morphology of habenular cells expressing insulin and to gain insight into the processing of human proinsulin in these cells. With pre-embedding immunocytochemistry, cells with no axo-somatic synapses were labelled, in agreement with the previous description of the medial habenula neurons, which were shown to display axo-dendritic synapses but hardly any axo-somatic ones [32].

We observed labelling precised at the sites of exported protein synthesis, *ie* the endoplasmic reticulum and Golgi apparatus cisternae. These observations suggest that the cellular processing of habenular proinsulin through the endoplasmic reticulum and Golgi apparatus might be identical to the processing demonstrated in pancreatic β cells; however, neither labelled Golgi vesicles nor secretory granules were found. Proinsulin cleavage in insulin and C-peptide was found to occur in the secretory granules of endocrine β cells [29]. Furthermore, processing of proinsulin was not obtained in a transfected fibroblast cell line, a cell type devoid of secretory granules, whereas the AtT-20 cell line, derived from mouse anterior pituitary cells, expressed a human proinsulin cDNA, processed proinsulin into insulin-like material and secreted it [28]. Here, secretory granules were not seen in habenular cells and it is unlikely that habenular proinsulin is cleaved and insulin secreted by the same regulated secretory pathway.

We cannot exclude possible constitutive secretion of (pro-)insulin, which might, in particular, affect the functioning of the medial habenula, since this structure has been shown to contain high levels of insulin receptor [34].

The habenular complex and its associated fiber systems, the stria medullaris and fasciculus retroflexus, form an obvious link between the forebrain and midbrain, and constitute one of the few regions in which the striatal and limbic systems converge. Medial habenula efferent fibers mainly project towards the interpeduncular nucleus and very scattered fibers project to the supracommissural septum and the medial raphe nucleus [15]. No HCP-immunolabelling was observed here in any efferent structures of medial habenula or in the fasciculus retroflexus, which suggests that habenular proinsulin is not transported along projection fibers. It is noteworthy that some short HCP, HPI or insulin-immunoreactive fibers were observed in medial habenula, suggesting that the *hins* gene might be expressed in connecting neurons and could thus affect the information process through the habenular cholinergic pathway. Since neither contact between cholinergic and SP–containing neurons of the medial habenula nor cholinergic projections from the medial to lateral habenula have ever been described, it seems unlikely that cells expressing the *hins* gene could interfere with neural pathways passing through the lateral habenula or through the substance P-containing neurons of the medial habenula.

In any case, the use of the Δ-168 transgene as a clue about the expression of the endogenous insulin genes in the brain remains to be tested with a more sensitive method than immunocytochemistry, such as RT-PCR analysis of RNAs extracted from microdissected medial habenulae. The generation of a medial habenula specific promoter from the human insulin gene could be of use in the construction of hybrid genes and the exploration of insulin action on habenular neurons.

Further studies are in progress to clarify habenular proinsulin processing and its possible secretion, and to investigate behavioral and physiological changes ascribable to the habenular expression of the human insulin gene.

Acknowledgements

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◀ Fig 7. Ultrastructural detection of human C-peptide in habenular cells. The immunoreaction occurs in cells displaying an indented nucleus (a). It affects discrete sites of endoplasmic reticulum (c) including the nuclear envelope (b, arrows) and Golgi saccules (d, arrows). No secretory granules can be seen. Scale bars: a, 1 μm; b, c, d, 0.5 μm.

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Cell-specific Expression of the Human Gonadotropin-releasing Hormone Gene in Transgenic Animals*

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We have previously demonstrated that 1131 base pairs (bp) of the human gonadotropin-releasing hormone (hGnRH) gene promoter can target simian virus 40 T antigen expression to GnRH neurons in transgenic mice. In these animals, GnRH neurons were transformed before they migrated to their final location in the rostral hypothalamus, complicating an analysis of cell-specific expression. To localize regions of the hGnRH promoter that are important for cell-specific expression, we created transgenic mice with various 5′-flanking regions of the hGnRH gene fused to the luciferase reporter gene. When 3828 or 1131 bp of the hGnRH promoter 5′-flanking DNA were used (−3828/+5LUC and −1131/+5LUC, respectively), luciferase expression in adult transgenic mice was observed in the rostral hypothalamus and olfactory tissues, regions which have been shown to be loci of GnRH-expressing neurons. Luciferase expression was not observed in other brain or peripheral tissues. Double-labeled *in situ* hybridization further demonstrated that luciferase expression was invariably colocalized with GnRH expression. When transgenic animals were created with a construct consisting of 484 bp of the hGnRH 5′-flanking DNA fused to the luciferase gene (−484/+5LUC), luciferase expression was not observed in the hypothalamus or in olfactory tissues. This is the first report localizing DNA sequences responsible for cell-specific expression of the GnRH gene *in vivo*.

The neurohormone gonadotropin-releasing hormone (GnRH)[1] is a decapeptide that is released from the hypothalamus and regulates the synthesis and release of the pituitary gonadotropins. It has become increasingly clear that the precise coordinated release and expression of GnRH are essential for proper function of the mammalian reproductive system. For example, patients with Kallmann's syndrome do not appear to contain GnRH neurons in the appropriate locations within the hypothalamus due to a failure of the GnRH neurons to migrate from the olfactory placode during development, which manifests as hypogonadotropic hypogonadism (1–3). Until recently, the study of the intracellular mechanisms regulating the release and expression of GnRH has been complicated by both the low numbers of GnRH neurons, as few as 800 in the mouse brain (4), and their scattered distribution from the hypothalamus to the olfactory bulb (5, 44).

In humans the GnRH gene consists of four exons and three introns (6–11), and its expression is limited to a small number of neurons in the brain, to gonadal and mammary tissue (12), and to human placental tissue (10, 13). Interestingly, GnRH expression displays a tissue specificity since the initiation site for placental GnRH expression is different than the initiation site for hypothalamic GnRH expression (10, 13). Recently, transgenic technology has given researchers the tools to study physiological and molecular aspects of the GnRH neuron. By targeting the SV40 T antigen oncogene to mouse GnRH neurons using the rat (GT1 cells (14)) or human (GN11 and NLT cells (15)) GnRH promoter, it has been possible to obtain a number of transformed GnRH-releasing cell lines. These cells possess a neuronal morphology, express and release GnRH, and respond to secretagogues in a manner consistent with that seen in GnRH neurons *in vivo* and *in vitro*. These cell lines have proven to be invaluable for studying the regulation of GnRH expression (16), processing (17), and release (18, 19) and for examining the electrophysiological properties of GnRH neurons (20, 21).

It is not clear at this time, however, what transcription factors or cis-regulatory elements are important for initiating and regulating the hypothalamic expression of hGnRH. The studies performed by our group using −1131 bp of the hGnRH promoter to target tumorigenesis in transgenic mice had appeared to transform only GnRH neurons (15), suggesting that this region of the hGnRH promoter was sufficient to direct gene expression to the GnRH neuron. However, since these neurons may not have completely differentiated before they were transformed and did not migrate to their final location in the forebrain, it was unclear whether this portion of the hGnRH promoter was actually the minimal promoter needed to appropriately direct and regulate the normal expression of GnRH.

We therefore sought to target expression of the luciferase reporter gene (22) with fragments of the promoter for the hGnRH gene. The luciferase gene is an extremely sensitive reporter, which is a quality necessary for use in the study of the physiological and developmental regulation of the sparsely distributed GnRH neurons. By linking various portions of the GnRH promoter to a luciferase reporter and creating transgenic animals with these constructs, it has been possible to examine the anatomical pattern of expression of luciferase as an assay of hGnRH promoter activity. Series of anatomical and physiological analyses have illustrated that luciferase expression that is driven by a sufficient segment of the hGnRH promoter is correlated with the expression of the endogenous GnRH gene. These studies localize a cis-regulatory region between −1131 and −484 bp upstream of the GnRH gene, which is essential for the appropriate expression of GnRH in the

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[1] The abbreviations used are: GnRH, gonadotropin-releasing hormone; h, human; bp, base pair(s); OVLT, organum vasculosum of the lamina terminalis.

brain. Additionally, these studies demonstrate that appropriate gonadal steroid regulation of GnRH expression requires gene elements present within 3828 base pairs of the hGnRH gene.

EXPERIMENTAL PROCEDURES

Transgenic Animal Construction—Transgenic animals were constructed by injecting a purified linear DNA fragment containing a GnRH/LUC construct free of vector DNA sequences into fertilized mouse oocytes as described previously by Radovick *et al.* (15). The hGnRH promoter fragments contained a variable amount of 5′-flanking DNA, but each had a common 3′ end point at +5 bp. Oocytes were then transferred into pseudo-pregnant foster mothers. Transgenic animals were identified by Southern blot analysis with a 1.2-kilobase pair LUC probe consisting of the coding sequence of the LUC gene using standard procedures. Briefly, *Eco*RI-digested mouse tail DNA was separated on a 0.8% agarose gel, transferred to GeneScreen Plus hybridization transfer membrane (DuPont NEN), UV cross-linked for 1.5 min, and placed into hybridization solution (46% formamide, 0.91 M NaCl, 9.1% dextran sulfate, and 1.8% SDS) for at least 5 h at 42 °C. Probe (1,000,000 cpm/ml hybridization solution) was mixed with 100 μg of salmon sperm/ml of hybridization solution, heat denatured for 5 min, and added to the hybridization solution overnight at 42 °C. The blots were then rinsed three times with 2 × SSC and washed for 30 min with 2 × SSC and 1% SDS at 65 °C.

Luciferase Assay of Animal Tissues—Animals were sacrificed by CO_2 narcosis and cervical dislocation. The rostral hypothalamus was dissected in a single fragment consisting rostrocaudally of tissue from the anterior edge of the mammillary bodies to a point just anterior of the optic chiasm, 1 mm laterally beyond the lateral aspect of the median eminence and 3 mm dorsally. The olfactory tissue was dissected to include both olfactory bulbs and the tissue rostral to the hypothalamic section; again, the dorsal incision was 3 mm deep. The caudal hypothalamus included the region from the rostral aspect of the mammillary bodies to a point 1 mm caudal to the mammillary bodies and with the same dorsal and lateral parameters as those taken for the rostral hypothalamus. Small sections were taken of the remaining tissues with less care taken to consistently target a specific intra-anatomical location within each organ or tissue. In most cases, however, whole regions were removed for homogenization. Tissues were removed and placed in 1 ml of lysis buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1% Triton X-100, and 1 mM dithiothreitol) homogenized with a Polytron tissue homogenizer (Brinkmann Instruments). 300 μl of the homogenate was then removed and centrifuged for 5 min at 15,000 × *g*, and 200 μl of the supernatant was assayed for luciferase activity.

Luciferase assays were done in a Lumat LB9501 luminometer (Berthold Systems Inc., Pittsburgh, PA). Samples were injected with 100 μl of luciferin (0.75 mM) and 100 μl of assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM KPO_4, 3 mM dithiothreitol, and 3 mM ATP), and luminescence was measured for 20 s in relative light units. The relative light units were then normalized for sample size by correcting for protein content. Protein assays were done using a Bio-Rad protein assay and bovine serum albumin standards.

Histology—Double-labeled *in situ* hybridization histochemical analyses of the hypothalamus and the organum vasculosum of the lamina terminalis (OVLT) of four different lines of −3828/+5LUC transgenic animals (three males and one female) and two different lines of −1131/+5LUC transgenic animals (one male and one female) were performed using antisense oligonucleotide probes to mouse GnRH and luciferase message. A labeled digoxigenin oligonucleotide (1 μl of digoxigenin/slide) corresponding to the decapeptide portion of the mouse GnRH gene was used to label GnRH mRNA, and three ^{35}S-labeled (250,000 cpm/section, 750,000 cpm/slide) 48-mer oligonucleotides against luciferase (bases 1464–1511, bases 321–368, and bases 1152–1105 (22)) were used as probes to label luciferase expression.

Sex Steroid Treatments of Transgenic Mice—Animals were gonadectomized under light avertin anesthesia (100% avertin = 10 mg of tribromyl ethanol in 10 ml of *tert*-amyl ethanol). In males, the testes were removed through a ventral incision made in the abdomen. Sham castrated animals received a ventral incision, but the testes remained intact. In females, the ovaries were removed through a dorsal incision, and sham ovariectomized animals also received a dorsal incision. Males received a pellet of either testosterone (5 mg/pellet) or cholesterol (5 mg/pellet) while females received a pellet of 17β-estradiol (0.5 mg/pellet) or cholesterol (5 mg/pellet), which was inserted dorsally with a 10-gauge trochar. All steroid pellets and the trochar were obtained from Innovative Research of America (Toledo, OH). The incisions were sutured, and the animals were allowed to recover for 2 weeks. Animals were checked daily to ensure that the wounds were healing properly.

TABLE I

Table displaying the number of founder animals constructed from each GnRh promoter-luciferase fusion construct, whether each transgenic type demonstrated tissue-specific expression of luciferase, and whether F1 or later generations have yet been evaluated

The histology and the effects of the sex steroids were examined in the same −3828/+5LUC founder line.

Construct used	No. of founders	Hypothalamic expression	F_1, or later, generations examined
−3828/+5LUC	3	Y	Y
−1131/+5LUC	5	Y	Y
−484/+5LUC	4	N	Y

Radioimmunoassays of β-Estradiol and Testosterone—Immunoreactive β-estradiol and testosterone were determined by radioimmunoassays using double antibody kits (ICN Biomedicals, Inc., Costa Mesa, CA). The level of sensitivity of the β-estradiol radioimmunoassay was 10 pg/ml with an intra-assay coefficient of variation (cv) of between 10 and 6% at the levels measured. The level of sensitivity was 100 pg/ml with a cv of 6% at the levels measured.

Developmental Expression of GnRH—Transgenic mice (−3828/+5LUC and −1131/+5LUC) were sacrificed with CO_2 at a variety of time points following birth (see Fig. 5). For prepubertal mice, the entire brain was homogenized and assayed for luciferase expression. For adult mice, luciferase expression from the hypothalamus and the olfactory cortex was combined to give the total value. Dissections were performed as described above.

Experimental Animals—Animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals.

RESULTS

A Region of the 5′ Flank of the hGnRH Gene Is Essential to Target Expression of a Reporter Gene in Transgenic Mice to the Hypothalamus—Transgenic animals were created using either −3828, −1131, or −484 bp of the hGnRH gene with a common 3′ end point of +5 fused to the luciferase reporter gene (−3828/+5LUC; −1131/+5LUC, and −484/+5LUC, respectively). Table I indicates the number of founder animals from each line that was obtained and the offspring that have been evaluated in this study. At least three founder lines from each construct were obtained, and these lines were bred to generate F1 and later generations. Fig. 1*a* illustrates the luciferase activity present in selected tissue extracts from a representative animal bearing the −3828/+5LUC transgene. Luciferase activity, when corrected for protein concentration in the tissue extract, was detected in hypothalamic and olfactory tissues but not in cortical, cerebellar, or other non-neural tissues. Expression in the hypothalamus was at least 50-fold greater than in any peripheral tissues. Non-transgenic animals (not shown) had no significant luciferase activity in these same tissues, indicating the specificity of this reporter gene assay for detecting GnRH gene expression in these tissues.

Similar results were obtained with the −1131/+5LUC construct. In a representative animal shown in Fig. 1*b*, both hypothalamic tissue and to a lesser extent olfactory tissue expressed the transgene at levels equal to or greater than the −3828/+5LUC construct. These data indicate that DNA sequences between −3828 and −1131 bp are not required to direct tissue-specific expression of the human GnRH gene to the hypothalamus and also are not required for basal expression of this transgene in mice. In contrast, the −484/+5LUC construct is not expressed in either the hypothalamus or the olfactory lobes, but expression was found in a mesenteric tumor (Fig. 1*c*). These results indicate that DNA sequences between −1131 and −484 bp of the promoter are essential to direct appropriate tissue-specific expression of the hGnRH gene in transgenic mice to rostral brain regions and suggest that dis-

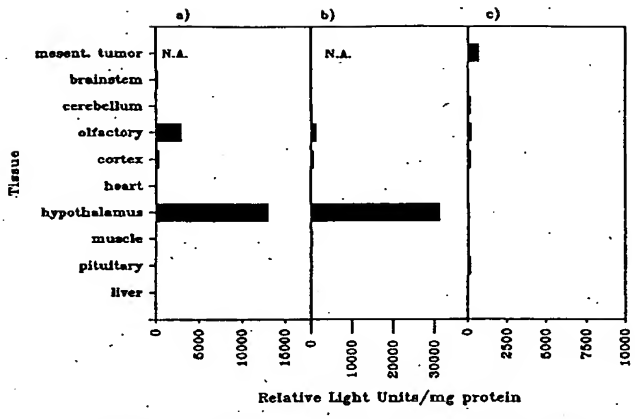

FIG. 1. **Relative luciferase activity of tissues taken from representative transgenic mice containing the (*a*) 3828/+5LUC (one of eight), (*b*), 1131/+5LUC (one of five), or (*c*) −484/+5LUC (one of six) transgene.** Luciferase activity was corrected for total protein in each tissue studied. Note the change in scale.

FIG. 2. **Relative luciferase activity of regions dissected from male (seven animals) and female (six animals) −3828/+5LUC mice.** Olfactory lobe (*open boxes*), preoptic area (*filled boxes*), and caudal hypothalamic (*stippled boxes*) tissues were examined. Luciferase activity was corrected for total protein content and is reported as the mean ±S.E. RLUs, relative light units.

crete cis-acting regulatory regions controlling GnRH gene expression are located in these DNA sequences.

Human GnRH Promoter DNA Fragments Are Essential to Target Luciferase Expression to GnRH Neurons—To further localize transgene expression of the −3828/+5LUC construct, the preoptic area, more caudal hypothalamic tissue, and the olfactory lobes were excised and assayed separately. Fig. 2 demonstrates that the majority of expression in either age-matched male or female adult animals was localized to the preoptic area. In keeping with expression of the GnRH gene in the mouse, more caudal regions of the hypothalamus and olfactory cortex also expressed significant luciferase activity.

To confirm that luciferase expression was localized to the GnRH neuron, *in situ* hybridization histochemical analysis of the rostral hypothalamus was performed in transgenic animals. Antisense probes to luciferase and mouse GnRH cDNA were hybridized to tissue sections. Fig. 3 is a representative tissue section through the OVLT of an animal bearing the −3828/+5LUC transgene. Colocalization of GnRH (digoxigenin-labeled probe, *purple*) and luciferase (^{35}S-labeled probe, *silver grains*) is demonstrated. Luciferase and GnRH probe hybridization colocalized to neurons in the OVLT and to a lesser extent in the caudal hypothalamus and olfactory lobes, appropriate regions for GnRH expression (5, 44). These studies demonstrated that luciferase and GnRH mRNA are consistently coexpressed in the same neurons and that physiologically relevant regulation of endogenous GnRH gene transcription can be evaluated by measurement of the luciferase reporter construct expression.

Sex-specific Differences in Regulation of the hGnRH Gene in Transgenic Animals—8- to 10-week-old adult mice from the same founder line underwent gonadectomy or sham surgery. 2 weeks after surgery, gonadectomized male and female animals were given replacement testosterone or estrogen, respectively. 14 days after replacement, animals were sacrificed and hypothalamic tissue was assayed for luciferase activity. Fig. 4, *a* and *b*, shows hypothalamic luciferase activity from −3828/+5LUC transgenic adult female and male animals, respectively, after sham surgery, gonadectomy, or gonadectomy and sex steroid replacement. Fig. 4*a* demonstrates that gonadectomy in females is associated with a significant increase in hypothalamic luciferase activity, and chronic estrogen replacement therapy resulted in a suppression of luciferase activity. In the olfactory lobe, gonadectomy also increased luciferase activity, and estrogen replacement returned luciferase activity back to control levels. These data suggest that estrogen, acting either directly on the GnRH neuron or indirectly via interneuronal pathways, can modulate the transcriptional activity of the hGnRH gene.

The data also demonstrated that castration of males resulted in some increased luciferase expression when compared with sham castrated males. Testosterone-treated castrated males expressed luciferase at levels not significantly different from those of sham castrated animals. These results suggest that testosterone may have inhibitory effects on GnRH transcription in male mice, but its effects were not as profound as those of estrogen in female animals. This sex difference is unlikely to be attributable to an insufficient efficacy of the testosterone treatment *versus* the estrogen treatment since radioimmunoassay measurements of serum testosterone and β-estradiol indicate that levels in steroid capsule-treated animals were between 6- and 22-fold higher than those seen in intact animals.

Developmental Regulation of the hGnRH Gene in Transgenic Animals—To understand how GnRH gene transcription varies with development, hypothalamic expression of luciferase was measured in transgenic animals bearing the −3828/+5LUC or the −1131/+5LUC transgene through development. Fig. 5 demonstrates hypothalamic luciferase activity during development of the −1131/+5LUC animals. Note that luciferase activity rises during the first few days of life and peaks around day 10, approximately 10–15 days before the first external signs of puberty in the mouse. Hypothalamic luciferase activity subsequently falls to adult levels after day 18. Therefore, peak luciferase activity, which is an indirect measure of GnRH gene transcription, is noted significantly before the onset of puberty in these animals. Similar results were obtained in −3828/+5LUC transgenic animals (data not shown). These data indicate that GnRH gene transcription can be modulated during development by as little as a −1131 to +5 bp of the hGnRH promoter and suggest that cis-acting elements mediating these developmental responses are located within this promoter region.

DISCUSSION

The appropriate expression and release of GnRH from neurons in the hypothalamus are vital for the elaboration of normal reproductive function in mammals. In the mouse, presumptive GnRH neurons arise from cells in the olfactory

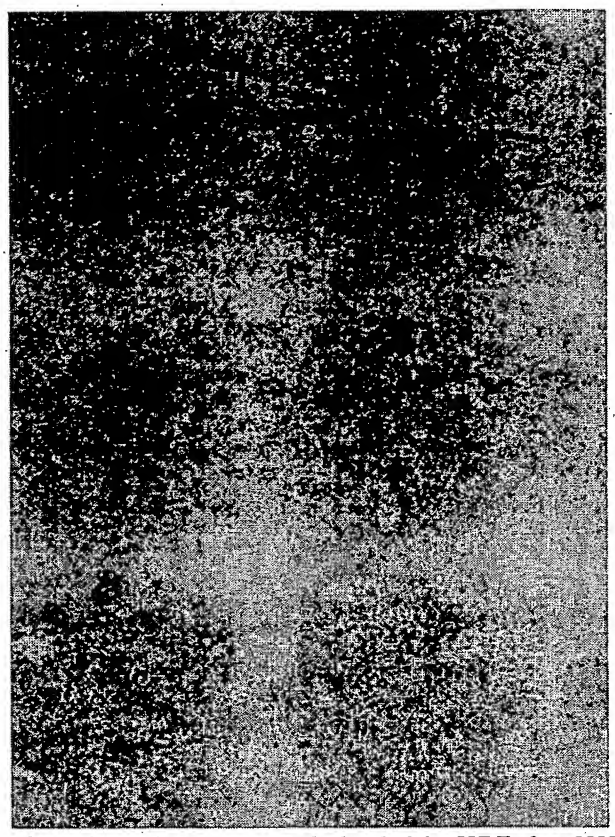

FIG. 3. **Coronal section cut at the level of the OVLT of a −3828/+5LUC transgenic mouse (one of four examined) and stained for GnRH and luciferase expression by *in situ* hybridization.** GnRH expression is indicated by *purple-staining* digoxigenin-labeled probe, and luciferase expression appears as *silver grains* (^{35}S-labeled probe). All cells that expressed GnRH also expressed luciferase, and all cells that expressed luciferase also expressed GnRH. Two −1131/+5LUC animals were examined as well, and the results were the same.

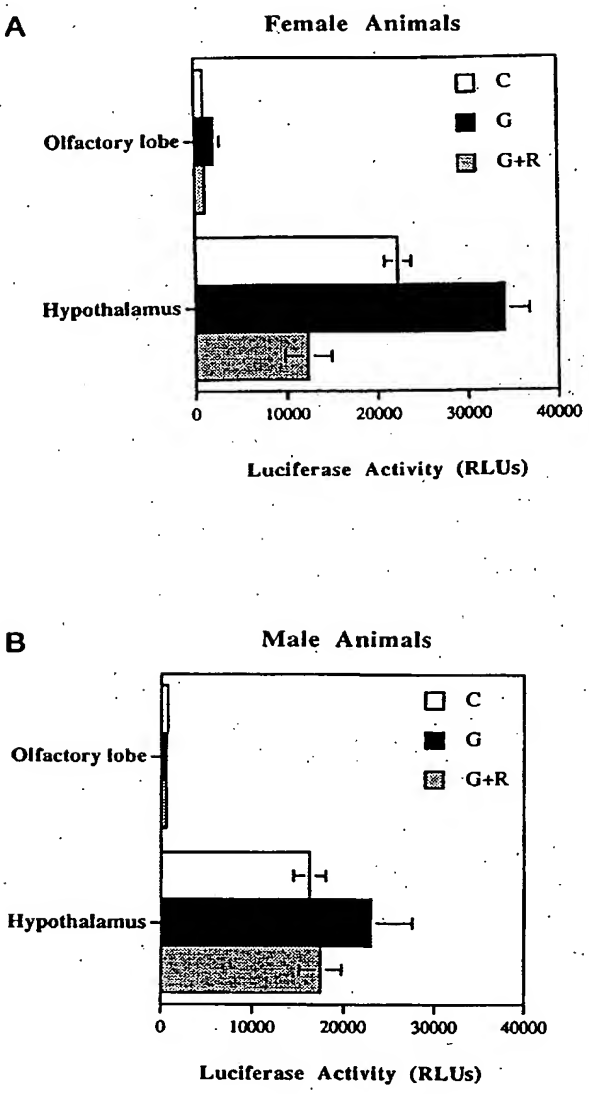

FIG. 4. **Relative luciferase activity of female (*A*) and male (*B*) −3828/+5LUC animals sham gonadectomized (*C*), gonadectomized (*G*), or gonadectomized and steroid replaced (*G* + *R*) (three to seven animals were in each group).** Olfactory cortex and hypothalamic tissue were examined separately and are displayed individually. RLUs, relative light units.

placode. They migrate during development to their final location in the hypothalamus. The initiation of GnRH expression starts during this period of cellular migration at about prenatal day 10 (4). In the adult, GnRH expression is located primarily in the rostral hypothalamus with additional neurons located in the migratory pathway within olfactory regions. In the present study, we have used the luciferase reporter gene as a cellular marker for expression and fused it to varying segments of the GnRH promoter. Both a −3828 to +5- and a −1131 to +5-bp region of the promoter were essential to direct expression of a luciferase reporter gene to hypothalamic neurons and to a lesser extent to neurons of the olfactory cortex (Fig. 1, *a* and *b*, respectively). However, a −484 to +5-bp fragment of the promoter did not target luciferase expression in the hypothalamus or olfactory cortex (Fig. 1*c*). Therefore, we have demonstrated that tissue-specific expression of the GnRH gene is mediated by a 647-bp fragment (−1131 to −484 bp) of the proximal promoter of the human GnRH gene.

Cell-specific expression of the luciferase reporter gene was demonstrated with double-labeled *in situ* hybridization (Fig. 3). Analysis of histological sections revealed that GnRH expression in the −3828/+5LUC animals and luciferase expression were colocalized in rostral hypothalamic tissue. GnRH staining and luciferase staining were never observed independently of each other. There were very low but detectable levels of luciferase expression in other tissues, and it is possible that expression of luciferase in non-hypothalamic tissue may have occurred in cells that were not expressing GnRH. However, these data confirm that cell-specific expression of hGnRH gene has been obtained in transgenic animals.

Recent work in the GT1 cell line, a mouse GnRH cell line that was developed by targeting the SV40 large T antigen to GnRH neurons with the rat GnRH promoter, found that cell-specific expression in the rat may require cis-regulatory regions more 5′ to what is needed to target the GnRH gene using the hGnRH promoter. The results obtained in the GT1 cell line have suggested that a cis-regulatory element located between −1863 and −1571 bp of the rat GnRH gene is necessary for the cell-specific expression of GnRH (26). By using DNase I footprinting and scanning replacement mutagenesis, several regions were found to be important for *in vitro* GnRH expression,

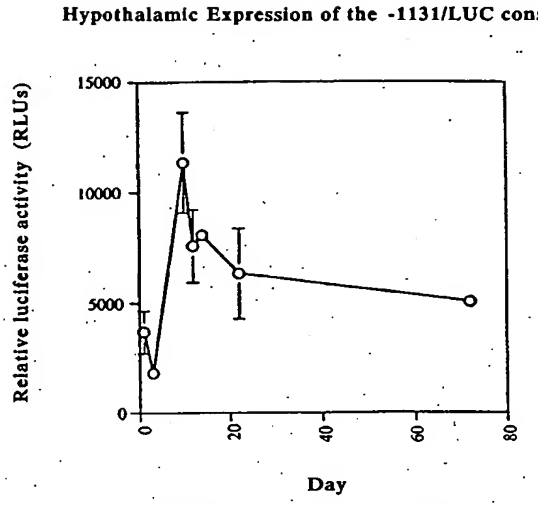

FIG. 5. **Total luciferase activity in the brain as a function of age in −1131/+5LUC transgenic animals.** Animals were sacrificed at various times after birth, and either whole brain (prepubertal) or hypothalamic and olfactory tissues (adults, the two values were added together for the total value) were assayed for luciferase activity. Data are the mean ± S.E. of three to eight individual determinants at the indicated time points.

and it was postulated that several different transcription factors might interact at these sites to regulate gene expression. While the rat enhancer is in a different location than the cell-specific element in the hGnRH gene, there is significant homology (54%) between the cis-regulatory elements in the rat gene and the region of the human GnRH promoter demonstrated in the present studies to be necessary for the cell-specific expression of GnRH (−1131 to −484 bp upstream of the hypothalamic transcriptional start site). It is unclear at this time whether this discrepancy in localization of cell-specific elements between the human and rat GnRH genes represents a species-specific difference.

Interestingly, the hGnRH cell-specific element contains several consensus binding sequences for POU homeodomain proteins, which in other systems have been demonstrated to act as cell-specific transcriptional activators (23, 24). Specifically, Brn-2 and Brn-3 sites were noted between −959 and −493 bp of the hGnRH gene. The corticotropin-releasing factor gene contains several Brn-2 DNA-binding sites, and Brn-2 has been shown to regulate the expression of the corticotropin-releasing factor gene (25). There are also two consensus Oct-1/Oct-2-binding sites located between −838 and −735 bp. Oct-1 has been proposed as an activator of immunoglobulin gene expression, and Oct-2 appears to be ubiquitously expressed (27, 28). Recently, Oct-1 has been shown to bind to the enhancer in the rat GnRH gene and increase gene transcription (29). Lymphocytes have been reported to express GnRH, and this site may function as a cis-acting element controlling expression in these cells (30). At this time, however, it is unclear whether these particular sites are involved in regulating expression of the hGnRH gene.

There is great debate concerning the effects of the gonadal steroids on GnRH expression. It has been reported that estradiol inhibits (31, 32), has no effect (33–36), stimulates (37–41), or has variable effects (42) on GnRH expression. The variable results in females are most likely due to differences in the experimental paradigms used and to the well characterized positive and negative feedback effects that estradiol has demonstrated to exert on GnRH neurons. However, most studies with chronic estrogen treatment *in vivo* suggest that GnRH expression is inhibited. Our results in the present study demonstrate that β-estradiol decreased expression of GnRH gene transcription, as measured by the luciferase reporter gene. Luciferase expression in females 2 weeks after ovariectomy was significantly higher than in intact females. When ovariectomized females were treated with estradiol, luciferase expression values were significantly lower than those seen in either gonadectomized or intact animals. These data indicated that estrogen can modulate the hGnRH gene promoter through either a direct action on the GnRH neuron or via other neuronal connections and that estrogen-responsive elements are contained within 3828 base pairs upstream of the hGnRH gene. There has also been a divergence of opinion regarding the effect of testosterone on GnRH expression (32, 34). Our results demonstrate that testosterone also has a significant effect on luciferase expression in males, although this effect was less profound than the effects of estrogen in the females. It is not clear whether these differences are due to an inherent disparity between androgen- and estrogen-mediated regulation of GnRH.

The onset of GnRH expression in the mouse is seen in the olfactory pit between day 10 and day 11 of embryological life (4). The bulk of GnRH-expressing neurons then migrate to their final location in the rostral forebrain. After birth, GnRH expression in the rat was found to increase sharply some 20 days before the onset of puberty and to decline to more moderate levels thereafter (43). Results from the present study indicate that the hypothalamic expression of GnRH gene in the mouse, as expected, has a similar temporal pattern of expression as that of the rat with a sharp transient increase in luciferase expression observed at 10 days, which is about 10–15 days before the beginning of reproductive function of the mice in our colony. Increased expression of GnRH is probably important for the increased production and release of GnRH that may trigger the initiation of reproductive viability. Our results suggest, but do not prove, that the increase in GnRH expression observed prior to puberty may be mediated by an increase in GnRH gene transcription.

In conclusion, our results clearly demonstrate that cell-specific *in vivo* expression of the human GnRH gene can be obtained with a region of the promoter between −1131 and +5 bp. A further deletion of this gene localizes a cell-specific element of the hGnRH gene to a region between −1131 and −484 bp. The elements responsible for the functional regulation of GnRH gene expression within this region have not yet been elucidated, but this region contains several DNA-binding site homologies for Oct-1, Oct-2, Brn-2, and Brn-3. It is tempting to speculate that the POU homeodomain family of transcription factors may have a role in the regulation of GnRH gene expression in the hypothalamus. Further studies will be necessary to prove or disprove this hypothesis.

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Characterization of a Cerebellar Granule Cell-Specific Gene Encoding the γ-Aminobutyric Acid Type A Receptor α6 Subunit

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Abstract: The α6 subunit of γ-aminobutyric type A receptors is a marker for cerebellar granule cells and is an attractive candidate to study cell-specific gene expression in the brain. The mouse α6 subunit gene has nine exons and spans ~14 kb. The largest intron (intron 8) is ~7 kb. For a minority of mRNAs, a missplice of the first exon was identified that disrupts the signal peptide and most likely results in the production of nonfunctional protein. The gene is transcribed from a TATA-less promoter that uses multiple start sites. Using transgenic mice, it was found that the proximal 0.5 kb of the rat α6 gene upstream region confers expression on a β-galactosidase reporter gene. One founder gave rise to a line with cerebellar granule cell-specific expression, although expression varied with lobule region. Other founders had ectopic but neuron-specific expression, with β-galactosidase found in cerebellar Purkinje cells; neocortex, thalamus, hippocampus, caudate-putamen, and inferior colliculi. Thus, we have defined a region containing the basal promoter of the α6 subunit gene and that confers neuron-specific expression. **Key Words:** Granule cell—Cerebellum—Transgenic mice—Promoter—α6 subunit.
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γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain and elicits fast inhibition by opening ligand-gated anion channels termed $GABA_A$ receptors. These pentameric channels are formed from a large subunit gene family comprising α, β, γ, and δ subunits (Darlison and Albrecht, 1995; Stephenson, 1995). Different subunit combinations are found in different parts of the brain, depending on brain region and cell type (see, e.g., Persohn et al., 1992; Wisden et al., 1992; Fritschy and Möhler, 1995). How do neurons select which subsets of the $GABA_A$ receptor subunit gene family to transcribe? The $GABA_A$ receptor subunit gene that has the simplest expression pattern is α6, which is therefore a good starting point for analysis. The expression of the α6 gene is restricted to cerebellar granule cells and the related granule cells of the cochlear nucleus (Kato, 1990; Lüddens et al., 1990; Laurie et al., 1992*a,b*; Thompson et al., 1992; Zheng et al., 1993; Varecka et al., 1994; Nusser et al., 1996). No expression is found in other brain regions.

Here, we characterise the intron/exon pattern and the transcriptional start sites of the rodent α6 subunit gene; we then show, using transgenic mice, that the information required to confer neuron-specific expression can be contained in a 500-bp proximal promoter fragment.

MATERIALS AND METHODS

Screening mouse and rat genomic libraries

A BALB/c mouse liver genomic library constructed in λ Fix I (Sommer et al., 1990), a 129 mouse strain-derived E14 TG2a ES cell library constructed in λ2001 (Warren et al., 1994), and a Sprague–Dawley rat testis genomic library in λ Dash II (Stratagene, La Jolla, CA, U.S.A.) were screened with a full-length rat α6 cDNA probe (Lüddens et al., 1990). Filters were hybridized in 50% formamide/5× saline–sodium citrate (SSC) at 37°C and washed in 0.4× SSC at 65°C (1× SSC is 150 m*M* NaCl and 15 m*M* sodium citrate).

Characterization of the $GABA_A$ receptor α6 subunit gene

The upstream sequences were obtained on mouse BALB/c and rat genomic fragments, whereas the overall gene structure was determined on mouse 129SV genomic fragments.

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A.J. and S.B. contributed equally to this work.

The promoter sequences reported have been deposited in the EMBL database with accession nos. X97475 (mouse) and X97476 (rat).

Abbreviations used: GABA, γ-aminobutyric acid; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SSC, saline–sodium citrate; TM, transmembrane domain.

To identify intron/exon boundaries and regulatory regions of the gene, hybridizing DNA fragments were subcloned into pBluescript SK⁻ (Stratagene) and partially sequenced. Mouse α6 subunit gene intron sizes and intron/exon boundaries were confirmed by restriction mapping, sequencing, and polymerase chain reaction (PCR) analysis using pairs of exon-specific primers and mouse liver (129SV strain) genomic DNA. PCR products were cloned into pPCR-Script SK⁺ (Stratagene) and sequenced.

PCR primers were as follows: intron 1, mα6INT1S, 5′-TGCTTCTCCCCTGGCTCTTCATTA-3′, and mα6INT1A, 5′-TCATCTTCAAGTTGAGCTTGGGCA-3′; intron 2, mα6INT2S, 5′-GCGAATTCAGGCTAGAAAATGCCCAAGCTC-3′, and mα6INT2A, 5′-GCGAATTCCATCTCCACATCTGACACAGGTCC-3′; intron 3, mα6INT3S, 5′-GCGAATTCGGACCTGTGTCAGATGTGGAGATG-3′, and mα6INT3A, 5′-GCGAATTCGTGTCAGGAGTCCAGATCTTACTG-3′; intron 5, mα6INT5S, 5′-CACGCTTGTCCACTCAAGTTTGGGA-3′, and mα6INT5A, 5′-TATGATTTCAGTTTTAGGATAAGC-3′; intron 6, mα6INT6S, 5′-GGGCAAACAGTTTCTAGTGAGAC-3′, and mα6INT6A, 5′-GTGGAAATAGACTGTCATGATTAC-3′; and intron 7, mα6INT7S, 5′-GAGTCAGTCCCAGCAAGAACTGTC-3′, and mα6INT7A, 5′-GTGGTCATGGTTAAAACAGTGGTG-3′.

Rapid amplification of cDNA ends (RACE) analysis

The putative promoter region was deduced by determining the start site(s) of transcription of both the rat and mouse α6 subunit genes.

RACE. Two rounds of PCR with AmpliTaq (Perkin-Elmer, Foster City, CA, U.S.A.) were performed on RACE Ready cDNA (Clontech, Palo Alto, CA, U.S.A.) from both mouse and rat brain using nested antisense primers and an anchor primer according to the manufacturer's protocols (Clontech) (Apte and Siebert, 1993). The first round of PCR was performed with 5′ RACE Ready cDNA, using the anchor primer and the outside primer and 30 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min. An aliquot of this reaction was then reamplified for another 30 cycles under the same conditions except using the nested primer. PCR products were cut with *Eco*RI or *Eco*RI/*Bam*HI and ligated into pBluescript and sequenced.

Primers used for the mouse (Kato, 1990) RACE were as follows: mα6RACE1, 5′-AGTTAACCAATCTCATGGGGCAATC-3′ (outside primer used for first round of PCR; complementary to the region encoding amino acids DCPMRLVNF); and mα6RACE2, 5′-GCGGATCCTGTTTTGACTTCTGTTACAGCACCCCC-3′ (nested primer for second round of PCR; complementary to the region encoding amino acids GGAVTEVKTD; *Bam*HI site underlined).

Primers used for the rat (Lüddens et al., 1990) RACE were as follows: rα6RACE1, 5′-AGTTAACCAGTCTCATCGGACAGTC-3′ (outside primer used for first round of PCR; complementary to the region encoding amino acids DCPMRLVNF); and rα6RACE2, 5′-GCGAATTCTGTTTTGACCTCTGTTACAGCACCTCC-3′ (nested primer for second round of PCR; complementary to the region encoding amino acids GGAVTEVKTD; *Eco*RI site underlined).

RNase protection assay

Templates to generate cRNA probes (~220 and 240 nucleotides long for the mouse and rat, respectively) were made by performing PCR with Vent (New England Biolabs, Beverley, MA, U.S.A.) polymerase on mouse and rat genomic restriction fragments and directionally subcloning the products (using sites incorporated in the primers) as *Eco*RI (5′ end) and *Bam*HI (3′ end) into pBluescript KS⁻ to give pmα6RNAP1 (mouse probe plasmid) and prα6RNAP1 (rat probe plasmid). The probe was designed to span ~100 bp of nontranscribed DNA and 100 nucleotides of the 5′ untranslated region identified from the longest RACE product. Primers to generate the mouse probe, with *Eco*RI and *Bam*HI sites underlined, were as follows: mα6RNAP1, 5′-GCGAATTCGGCCTATGTAGGATTCC-3′ (upstream sense); and mα6RNAP2, 5′-GCGGATCCAGCCTCCTCTGACTGGT-3′ (downstream antisense). Primers to generate the rat probe were as follows: rα6RNAP1, 5′-GCGAATTCCCTTCTGTGAACCATAG-3′ (upstream sense); and rα6RNAP2, 5′-GCGGATCCGTCGTTTGTGGTCTTAG-3′ (downstream antisense). For the positions of these primers, see Fig. 4.

Antisense cRNA probes were generated by linearizing the plasmids with *Eco*RI and transcribing with T7 RNA polymerase and [α-^{32}P]UTP (800 Ci/mmol; DuPont NEN, Stevenage, U.K.) using an Ambion (Austin, TX, U.S.A.) Maxiscript kit. The transcribed polylinker of the pBS-KS plasmid between the T7 promoter and the *Bam*HI site added a further 50 nucleotides to the probe, giving a total probe length of 270 nucleotides for the mouse and 290 nucleotides for the rat. RNase protection assays were performed using an Ambion RPAII system according to the manufacturer's instructions. The probe was hybridized with mRNA overnight at 44°C [hybridization buffer: 80% formamide, 100 m*M* sodium citrate (pH 6.4), 300 m*M* sodium acetate (pH 6.4), and 1 m*M* EDTA]. Control reactions, incubated in parallel, were as indicated in the legend of Fig. 5. Samples were analysed on 5% acrylamide/8 *M* urea gels.

In situ hybridization

In situ hybridization on BALB/c mouse brain sections with ^{35}S-labelled oligonucleotides (36 mers) was as described (Wisden and Morris, 1994). Sections were hybridized in 50% formamide, 4× SSC, and 10% dextran sulphate at 42°C and washed in 1× SSC at 60°C. Controls were performed with a 100-fold excess of unlabelled oligonucleotide in the hybridization buffer. After ethanol dehydration, sections were exposed to Biomax MR film (Kodak, New Haven, CT, U.S.A.). Oligonucleotide sequences were as follows: mα6Exon1a/2, 5′-GAGCTTGGGCATTTTCTAGC-"I"-CTGGAGAAGTCTTCCA-3′ (hybridizes to novel RNA splice version: exon1a–exon2; "I" indicates the position of the splice junction; see Fig. 2); mα6Exon1, 5′-GAGCCAGGGGAGAAGCAAGACCATCCTATGGTTCTC-3′ [hybridizes to exon 1, encoding the originally published signal peptide, nucleotides +360 to +395 (see Fig. 2), and does not hybridize to the novel splice version, exon 1a–exon2]; mα6Pan, 5′-GACAGGCGTCGATTTTAAGATGGGCGTTCTACTGAG-3′ [exon 9-specific, transmembrane domain (TM) 3–TM4 loop region, amino acids LSRTPILKSTPV, detects all α6 mRNA forms (Kato, 1990)]; and mα6Start, 5′-TCCACGGCTAGTTTTGGCATGGACTTCGAATGGGCT-3′ [detects mRNAs originating near the most 5′ start site(s) of transcription, nucleotides +1 to +35 (see Fig. 4)].

Transgenic mice

A transgene construct using the pnLacF vector was prepared. The pnLacF cartridge is a promoterless β-galactosi-

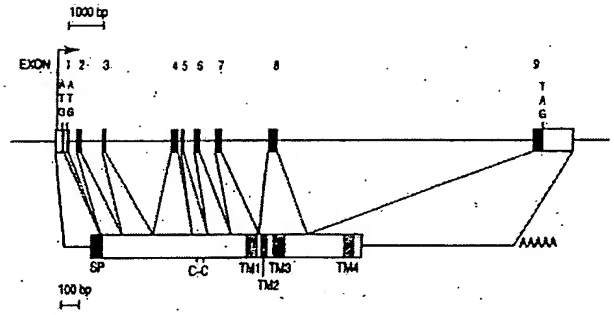

FIG. 1. Exon/intron structure of the mouse α6 GABA$_A$ receptor subunit gene mapped onto the mRNA. The arrow indicates the start site(s) of transcription. On the gene structure, open boxes indicate 5′ or 3′ untranslated regions; solid boxes indicate coding regions. SP, signal peptide; C–C, dicysteine loop structure.

dase (*lacZ*) gene that contains an intron, 3′ untranslated sequence, and a polyadenylation signal derived from the mouse protamine gene (Mercer et al., 1991). A linker encoding the SV40 nuclear localization signal peptide is fused to the 5′ end of the *lacZ* coding region to facilitate histochemical detection (Mercer et al., 1991). The construct rα6n-LacZ0.9 was derived from the rat α6 subunit gene. It contains ~500 bp of 5′ nontranscribed region (proximal promoter) and the complete 5′ untranslated segment (~350 bp), with the first methionine of the α6 coding region replaced with the *lacZ* coding region (see Fig. 6). A 900-bp PCR fragment with *Nco*I sites at both ends was generated using T3 and rα6NcoI primers, with a subcloned rat genomic fragment as a template: The 5′ *Nco*I site is a natural one adjacent to the *Bam*HI site, whereas the 3′ site was incorporated in the downstream rα6NcoI primer (5′-GAGAAG-CAACACCATGGTATGGTTCACTG-3′) to introduce an *Nco*I site at the start of translation of the α6 gene. The PCR product was cloned into the *Nco*I site of pnLacF and sequenced to verify the fidelity of the PCR.

Before oocyte injection, the transgene (see Fig. 6) was isolated from the pUC backbone of pnLacF by digesting with *Xba*I and *Hin*dIII. Transgenic mice (strain CBA/cba × C57/BL6) were produced as described (Hogan et al., 1994). Positive founders were identified by Southern blot analysis of tail genomic DNA digested with *Bgl*II. Membranes were probed with a *lacZ* restriction fragment isolated from the pnLacF vector (Mercer et al., 1991). Mice were perfused with 4% paraformaldehyde in phosphate-buffered saline. Brains and other selected organs (liver, kidney, and heart) were cut frozen on a sliding microtome and incubated with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) for 12–36 h at 37°C (Hogan et al., 1994). Sections were counterstained with neutral red.

RESULTS

Gene structure

The size of the mouse α6 gene is ~14 kb (Fig. 1 and Table 1). The gene contains nine exons. The smallest intron (intron 4) is just 75 bp, and the largest intron (intron 8) is 7.0 kb (Table 1). The gene structure conforms to that established for other members of the GABA$_A$ receptor gene family (reviewed by Harvey et al., 1994). For the α6 gene, exon 1 encodes most of the signal peptide. Exon 2 encodes the last six residues of the predicted signal peptide and the first 34 amino acids of the mature polypeptide; exons 3–7 encode the remainder of the extracellular domain. Exon 4 encodes the arginine residue at position 100 critical for determining the benzodiazepine pharmacology of the α6 subunit (Korpi et al., 1993*a*). Exon 5 encodes the dicysteine loop structure. Exon 7 encodes part of the extracellular domain, all of TM1 and part of TM2. Exon 8 encodes the rest of TM2, all of TM3, and part of the predicted large intracellular loop. Exon 9 encodes the remainder of the intracellular loop, TM4, and the carboxyl-terminus (Fig. 1).

Alternative splicing of exon 1

A RACE analysis (see below) of the 5′ end of the mouse α6 mRNA revealed that some transcripts were deleted in the region encoding the published signal peptide sequence (Kato, 1990; Lüddens et al., 1990). Three of 17 independent clones derived from the mouse RACE were found to have identical deletions when aligned to the genomic sequence. Alternative splicing of the α6 RNA around the region encoding the signal peptide occurs via differential 5′ splice donor sites. The novel 5′ splice donor (gt) site identified here is in the 5′ untranslated region of the mouse α6 subunit RNA at nucleotide position +297 and uses the 3′ splice acceptor site at the end of intron 1 (Table 1 and Fig. 2). At this exon/intron boundary (CAG/gtgatt), eight of nine nucleotides are in agreement with the extended 5′ donor splice site consensus sequence MAG/GTRAGT (where the boundary is denoted by a slash, M = A or C, and R = A or G) (Smith et al., 1989). It is predicted that when this alternative splice donor site is used, the majority of the signal peptide (MVLLLPWLFIILW) encoded by exon 1 will be skipped and replaced with the reading frame (MRNMKDLEDFSR) from exon 1a (Fig. 2), assuming that the first in-frame ATG of exon 1a (at position +252) can initiate translation.

Using an approach similar to that of Korpi et al. (1994), we confirmed the existence of the alternative spliced mRNAs around the signal peptide region by in situ hybridization to mouse brain sections, using an oligonucleotide (mα6Exon1a/2) that spans the splice junction. The mα6Exon1a/2 probe gives granule cell-specific hybridization (Fig. 3c). As expected, antisense oligonucleotides specific for the original signal peptide (exon 1) and the large intracellular loop region of the subunit (exon 9) also give granule cell-specific hybridization (Fig. 3a and b).

Because the exon 1a peptide contains no hydrophobic stretch of amino acids but instead contains alternating charged residues (Fig. 2), it is unlikely to function as a signal peptide sequence for targeting the subunit into the endoplasmic reticulum. The corresponding region of the rat 5′ untranslated region of the RNA does

TABLE 1. *Intron/exon organization of the mouse GABA$_A$ receptor α6 subunit gene*

1	TATTCTATG/gtaagaaga	0.225 kb	cacctttcag/GCTAGAAAA	2
1a[a]	CTTCTCCAG/gtgattgtc	0.39 kb	cacctttcag/GCTAGAAAA	2
2	GATTTGGGG/gtagtaaaa	0.64 kb	ttattttcag/GTGCTGTAA	3
3	GTGGAGATG/gtgagtaca	1.8 kb	ttctcttag/GAGTATACA	4
4	TACCATGAG/gtgagttgt	0.075 kb	tgtctgcag/GCTCACCAT	5
5	TTGGGAGCT/gtaagttac	0.29 kb	aaactacag/ATGCTTATC	6
6	CTAACACTG/gtaagaata	0.39 kb	ttccaacag/GTGAATATG	7
7	CTGTCTTTG/gtatgcctc	1.3 kb	ttatttcag/GAATCACCA	8
8	ATTGTTGTG/gtaattgtt	7.0 kb	ctttcttag/CATTCTGAC	9

Exon 1 encodes the original published signal peptide. The boundaries and nucleotide sequence of exon 1 are identical to the results of a partial analysis of the rat α6 gene (McLean et al., 1995). The 3′ (ag) boundary of intron 3 was assigned according to that found in the rat α6 gene (Korpi et al., 1994), with the published mouse α6 cDNA sequence assumed to result from a cryptic splice acceptor site usage giving rise to a 30-nucleotide deletion (Kato, 1990; Korpi et al., 1994).

[a] Exon 1a is assigned on the basis of the putative cryptic splice donor site in the 5′ untranslated region and RACE analysis.

not have the splice donor sequence (the critical "g" of the gt splice donor is replaced with "t" at nucleotide +307, and the first in-frame ATG is replaced with ATA, at nucleotide +274; see Fig. 4), and so it is probably safe to view the situation as an aberrant splice.

Mapping the transcription start site(s)

The sequence of both the rat and the mouse genes upstream of the first methionine is shown (Fig. 4). This includes the limit of the published 5′ untranslated regions of the cDNAs (Kato, 1990; Lüddens et al., 1990) (marked as solid black triangles/arrowheads on Fig. 4) and extends into the novel sequence. The longest insert obtained from screening a specifically primed rat cerebellar cDNA library, where the cDNA was made by priming reverse transcriptase with antisense oligonucleotides derived from sequences in exon 1, indicated a

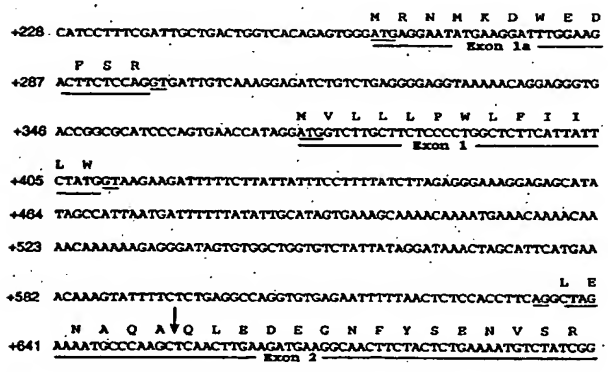

FIG. 2. Alternative splicing of the signal peptide-encoding exon 1 of the mouse α6 GABA$_A$ receptor subunit gene. The arrow indicates the predicted cleavage site of the signal peptide (Kato, 1990; Lüddens et al., 1990). The nucleotide numbering is according to Fig. 4 and starts at nucleotide +228 in the 5′ untranslated region.

possible start site of transcription 304 nucleotides longer than the published rat cDNA (data not shown; marked as a solid diamond on Fig. 4). In situ hybridization, on mouse brain sections using an antisense oligonucleotide (mα6Start) built to the 5′ end of the longest cDNA identified from the specifically primed library, confirms that this sequence is transcribed (Fig. 3d), but that it is either not abundant or alternatively that the 5′ cap region of the mRNA is less accessible for hybridization (see, e.g., Köhler et al., 1994).

To determine more accurately the start site(s) of transcription we performed RACE and RNase protection assays on mRNA derived from mouse and rat cerebelli. RACE analysis revealed a cluster of start sites in roughly the same region for both the mouse and rat genes (arrows on Fig. 4), which correlated with the presence of a single band of RACE product on ethidium bromide-stained agarose gels (data not shown). In all, 17 mouse and seven rat RACE products were completely sequenced. The majority (15) of the mouse RACE products were clustered in the sequence 5′-AATTATTGTCATTGCT-3′, with the two underlined nucleotides each having six independent RACE products assigned to them (Fig. 4, mouse nucleotides +63 to +78). Two of the rat RACE products also start within this cluster, and another two are found 10 and 20 bases further 5′ of this. Two mouse and one rat RACE product were found to start 3′ (10 and 20 bases, respectively) from the last nucleotide of the main cluster. To look for any longer (and rarer) products, the ligations of RACE products were colony screened with the mα6Start in situ hybridization oligonucleotide (see Materials and Methods), which is built to the most 5′ sequence of the longest cDNA identified from the specifically primed library. This identified two further RACE products (marked "+1." on Fig. 4), both starting at the same nucleotide. This nucleotide is 60–70 bases further upstream than the main cluster of start sites. The longest RACE product was arbitrarily used to define the sequence numbering as +1.

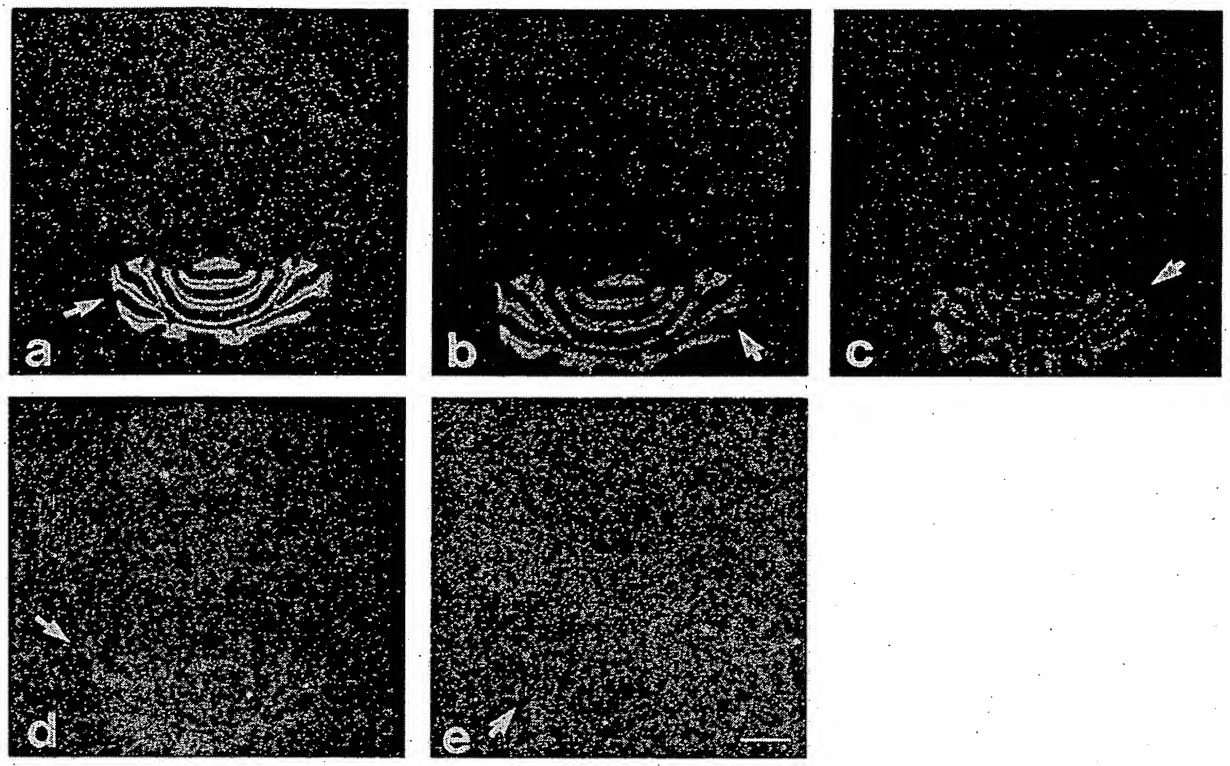

FIG. 3. X-ray film autoradiographs of in situ hybridization to adult BALB/c mouse brain horizontal sections using ^{35}S-labelled α6 exon-specific oligonucleotide probes. **a:** mα6Pan oligonucleotide (hybridizing to the intracellular loop region between TM3 and TM4, exon 9). This probe serves as a positive control. **b:** mα6Exon 1 oligonucleotide (specific for exon 1; see Fig. 2). **c:** mα6Exon1a/2 oligonucleotide (bridges exon 1a/exon 2 splice junction; see Fig. 2). **d:** mα6Start oligonucleotide, hybridizing to the most 5′ region of the α6 subunit mRNA. **e:** Control shows the ^{35}S-labelled mα6Exon1a/2 hybridized with an excess of unlabelled mα6Exon1a/2 oligonucleotide. Arrows indicate labelled granule cell layer of the cerebellum. Bar = 1.75 mm.

All RACE cDNA sequences obtained, with the exception of the splice variants that contained the deletion of exon 1 (see Alternative splicing of exon 1), were colinear with the genomic sequences. No novel sequence was obtained for the 5′ end of any of the mRNAs, suggesting that there were no unidentified introns between the start site(s) of transcription and the beginning of the coding sequence, i.e., the entire 5′ untranslated region and the signal for the initiation of translation are on one exon.

RNase protection on both rat and mouse cerebellar mRNA gave predominantly protected bands consistent with the RACE analysis (Fig. 5). The probes were designed to flank symmetrically (~100 nucleotides transcribed and protected/100 nucleotides untranscribed and unprotected) the two longest start sites identified by RACE (marked "+1" on Fig. 4). However, no protected fragments were seen in the 100-bp region, suggesting, in accordance with the frequency obtained from RACE analysis, that transcripts initiating from this site are rare (Fig. 5). Instead, for the mouse a strong doublet was seen at ~35–40 bp that matches the cluster of RACE products in this region (Figs. 4 and 5). In the rat, where the region used for the probe is shifted 20 nucleotides in the 3′ direction relative to the mouse, a predominant band was seen at ~50 bp, which is actually 20 nucleotides shorter than the rat RACE products. However, a fainter collection of larger bands was always present in the 70–90-bp region that would fit with both the mouse and rat RACE cluster. No protected fragments were found in mRNA isolated from forebrain or liver (Fig. 5). Together the RACE and RNase protection assays suggest that the α6 subunit gene promoter uses a cluster of start sites spanning a 100-bp region.

Sequence analysis of the proximal 5′ region

Approximately 0.5 kb of both the mouse and rat α6 subunit gene sequences was determined upstream of the transcription start site region (Fig. 4). Aligning the two sequences reveals an overall high level of conservation, as would be expected when comparing two rodent species. Both sequences are rich in AT residues, particularly in the area around −450 to −250 bp. The region just upstream of the mapped start sites lacks obvious TATA or CAAT boxes. Possible transcription

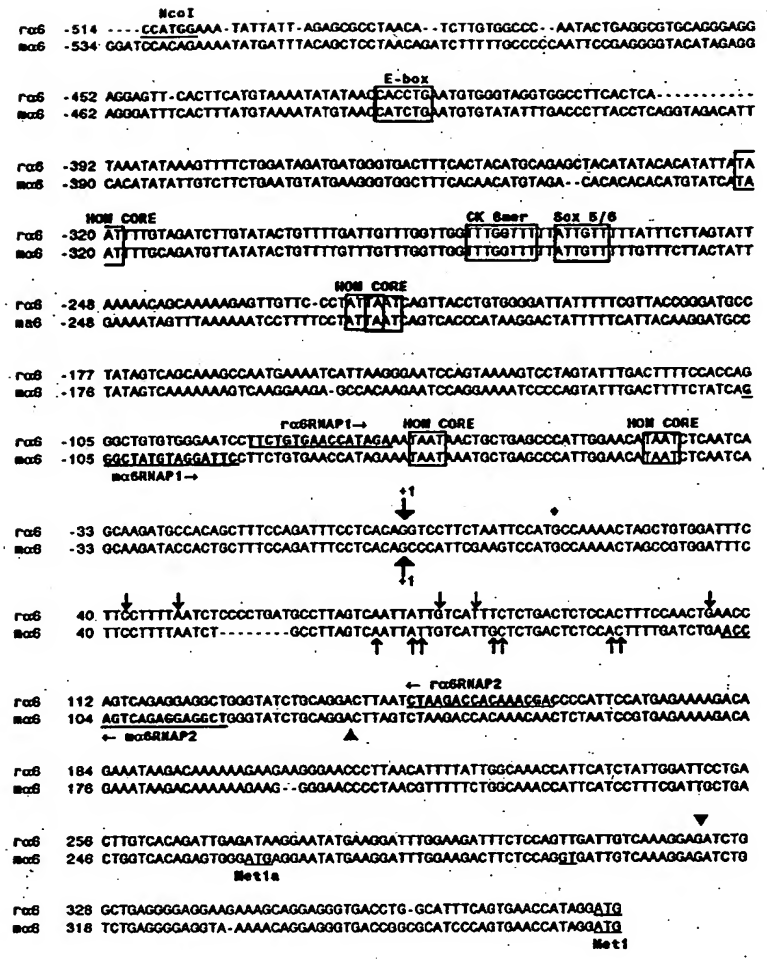

FIG. 4. Sequence alignment of the proximal promoter and 5′ untranslated regions of the mouse (m) and rat (r) α6 subunit genes. The solid triangles show the extent of the published cDNA sequence for the mouse (Kato, 1990) and the rat (Lüddens et al., 1990). Thin arrows show the 5′ limits of mRNAs determined by RACE. The thick black arrows mark the longest RACE products and were arbitrarily defined as +1. The solid diamond above the rat sequence marks the longest cDNA obtained from a specifically primed cerebellar cDNA library. Putative binding sites for transcription factors are boxed. The oligonucleotides (rα6RNAP1, rα6RNAP2, mα6RNAP1, and mα6RNAP2) used to generate probes for the RNase protection assays are underlined. Met 1 and Met 1a indicate reading frames of exon 1a and exon 1 (see Fig. 2). The *Nco*I site in the rat gene at position −514 used for constructing the transgene (see Fig. 6) is shown.

factor binding sites found to be present in corresponding regions of both mouse and rat α6 gene proximal regions, include five core consensus binding sequences for homeobox-containing proteins at positions (mouse sequence) −45, −71, −219, −221, and −322 [5′-TAAT-3′ (Ekker et al., 1994)], a CK8mer motif at position −273 [5′-AANCCAAA-3′, where N is any nucleotide (Blessing et al., 1987)], and a Sox5/6 binding site at position −265 [5′-AACAAT-3′ (Connor et al., 1995)]. An E-box 5′-CACCTG-3′ in the rat and 5′-CATCTG-3′ in the mouse is found at position −432 [consensus 5′-CANNTG-3′ (Weintraub et al., 1994)] (Fig. 4).

Transgenic mice: analysing the promoter regions

Because no known cell line expresses the α6 gene (Tyndale et al., 1994), we used a transgenic approach to assay the α6 promoter. We examined whether the proximal 500-bp upstream region of the rat α6 gene was capable of conferring correct spatial expression on a promoterless β-galactosidase (*lacZ*) gene when randomly integrated into the mouse genome (Fig. 6).

Fourteen independent founder mice were generated with the rα6nLacZ0.9 transgene. Of these, four expressed β-galactosidase. One founder (founder A) gave a virtually correct pattern, exhibiting predominantly cerebellar granule cell-specific expression (Fig. 7). Numerous clusters of nuclear-stained granule cells were found (Fig. 7A). Expression was not found in Purkinje cells, in stellate/basket cells in the molecular layer, or in the white matter tracts. Some scattered positive cells were noted in the inferior colliculi of line A (data not shown), but all other brain areas were negative (see, e.g., Fig. 8C). However, unlike the native α6 gene, expression of the transgene varied with the cerebellar lobules examined. Some folia had high numbers of positive cells (Fig. 7A). Others on the same section had relatively few or no positive cells (Fig. 7B). This pattern was reproducible in descendants bred from founder A. The other founders gave

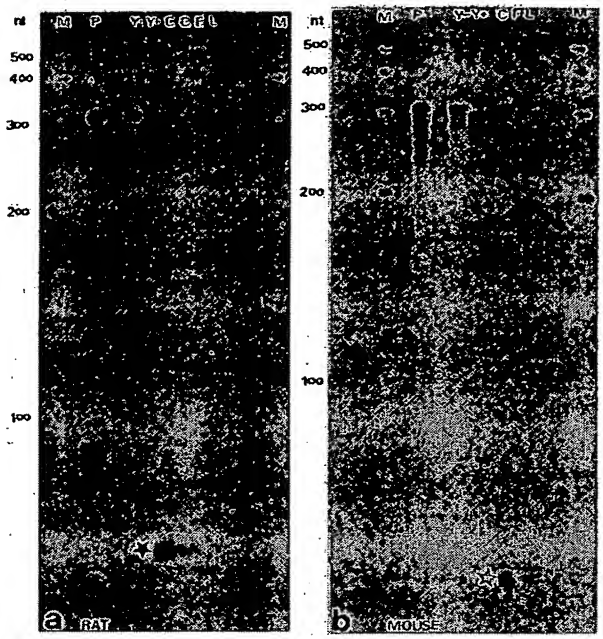

FIG. 5. RNase protection assay to analyse the start site of transcription of (a) rat and (b) mouse α6 subunit genes. M, transcribed RNA markers (Ambion); P, probe only; Y−, probe hybridized with yeast tRNA but no RNase cocktail added; Y+, probe hybridized with yeast tRNA and RNase cocktail added; C, F, and L, probe hybridized with cerebellar, forebrain, and liver mRNA (10 μg per lane) isolated from the rat and mouse, respectively. For the rat cerebellar mRNA, 10 (left-hand lane) and 5 μg (right-hand lane) were hybridized. Solid and open stars indicate the major protected bands. No protected bands were found for the forebrain and liver samples. nt, nucleotides.

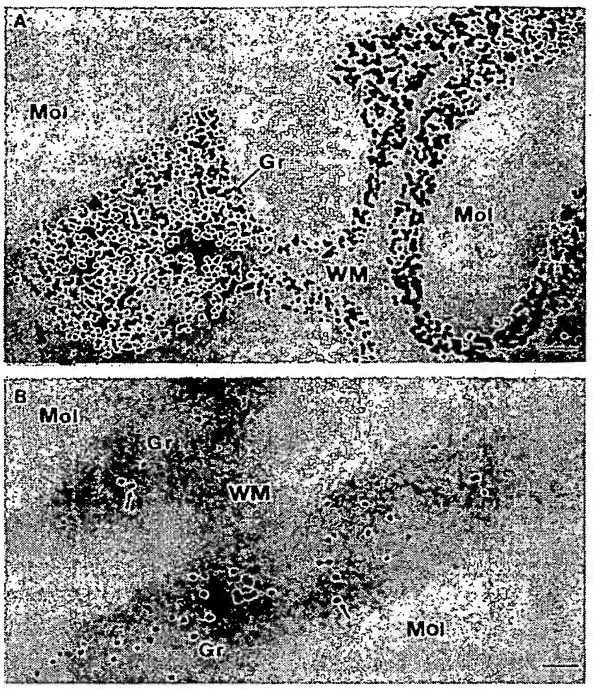

FIG. 7. Expression of the transgene rα6nLacZ0.9 in founder line A gave a correct expression pattern. A: Many granule cells with *lacZ*-positive nuclei. B: Other folia from the same section of cerebellum illustrated in A had far fewer positive cells (arrows). *LacZ* expression for this line was also seen in the inferior colliculi but in no other brain regions (see Fig. 8C). Gr, granule cell layer; Mol, molecular layer; WM, white matter. Bar = 120 μm in A and 45 μm in B.

neuronal but ectopic expression (Fig. 8). In founder B, strong nuclear staining was seen in the cerebellar Purkinje cells (Fig. 8A). There was no expression in the granule cell layer, stellate/basket cells, or white matter (Fig. 8A). In the forebrain, expression was particularly strong in the dentate granule cells of the hippocampus (Fig. 8B), the thalamus (Fig. 8B), and the caudate-putamen (Fig. 8D) with weaker expression observed in the neocortex. No glial expression was seen. In founders C and D, expression was seen only in the inferior colliculi (Fig. 8E). No *lacZ* expression was noted in white matter tracts or in the peripheral tissues examined (liver, kidney, and heart) of any animal.

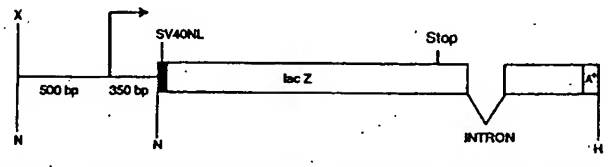

FIG. 6. Structure of the transgene rα6nLacZ0.9. The transgene consists of 500 bp of nontranscribed and 350 bp of 5′ untranslated sequence from the rat α6 GABA$_A$ receptor subunit gene fused to the *nLacZ* reporter gene (Mercer et al., 1991). Black shading indicates the nuclear localization signal, A$^+$ represents the polyadenylation signal. The arrow shows the start site(s) of transcription. Relevant restriction enzyme sites are *Hin*dIII (H), *Nco*I (N), and *Xba*I (X).

DISCUSSION

Many subtypes of GABA$_A$ receptor are found in the vertebrate CNS. Different neuronal and glial cell types transcribe different subsets of the multisubunit gene family, but it is not understood how this is achieved and regulated. The α6 gene expression is part of the terminally differentiated state of mature granule cells. The gene is switched on after the developing granule cells have migrated from the external granule cell layer into the internal layer (Laurie et al., 1992*b*; Korpi et al., 1993*b*; Kuhar et al., 1993; Zheng et al., 1993; Varecka et al., 1994). Ultimately, we wish to learn how this confined expression pattern is generated.

Gene structure and alternative splicing of GABA$_A$ receptor subunit genes

The organization of the α6 gene conforms to the general organization described for other subunit classes

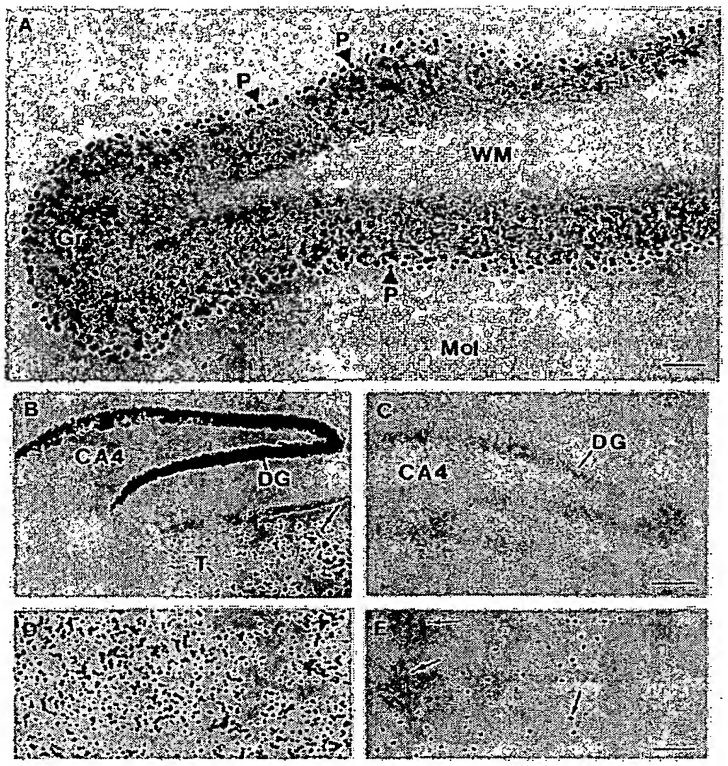

FIG. 8. Examples of ectopic neuronal expression of the rα6nLacZ0.9 transgene. **A:** Purkinje cells (P). Gr, granule cell layer; Mol, molecular layer; WM, white matter. **B:** Hippocampal dentate granule (DG) cells and thalamus. **C:** Hippocampus and thalamus of line A show the absence of transgene expression. **D:** Many positive cells in the caudate-putamen. **E:** Founder with strong expression of scattered cells in the inferior colliculus. Bar = 100 μm in A, 130 μm in C, and 50 μm in E.

in vertebrates and snails. $GABA_A$ receptor subunit genes contain nine principal exons, with intron–exon junctions conserved between different subunit types (reviewed by Harvey et al., 1994). In the chick and human α1 subunit genes, an intron of unspecified size interrupts the 5′ untranslated region (Bateson et al., 1995), and the rodent α6 subunit is different in this respect, with the 5′ untranslated region being on one exon. At ~14 kb, the α6 gene is in the same size range as the rodent δ-subunit gene (Sommer et al., 1990; Motejlek et al., 1994) and is considerably more compact than the β-subunit genes (mammalian β1 and β3, avian β4, and the snail "β-like" subunit gene), which are characterized by very large introns (Harvey et al., 1991; Kirkness et al., 1991; Lasham et al., 1991; Greger et al., 1995). The human β1 and chicken β4 genes both stretch over 65 kb, the snail "β-like" subunit gene is at least 40 kb (Harvey et al., 1991; Kirkness et al., 1991; Lasham et al., 1991), and the human γ3 subunit gene also spans at least 50 kb (Greger et al., 1995).

The exon 1 region of transcripts from the mouse α6 subunit gene is differentially spliced via alternative 5′ donor sites. The predicted consequence is that some (~17% as deduced by RACE analysis) of the α6 mRNA encodes a nonfunctional leader peptide. There is a precedent for alternative splicing of exon 1 of $GABA_A$ receptor subunit genes: A bona fide alternative signal peptide results from a differential splice for exon 1a of the human β3 subunit gene (Kirkness and Fraser, 1993). Another missplice of the mRNA encoding the rodent α6 subunit has been described with the alternative use of 3′ acceptor sites at the end of intron 3 (Korpi et al., 1994). This splice deletes 10 amino acids from the N-terminal extracellular domain and also results in a nonfunctional protein that fails to escape from the endoplasmic reticulum (Korpi et al., 1994; Stephen Moss, personal communication). Both alternative splices of the α6 RNA use differential splice donor and acceptor sites that cause small deletions/replacements of the polypeptide chain, and this same category of splicing has been observed for genes encoding other subunits in the nicotinic receptor subunit gene superfamily (see, e.g., Bateson et al., 1991).

The α6 gene proximal promoter region

We assayed the activity of the proximal 500-bp promoter region of the rat α6 gene linked to a β-galactosidase reporter cassette. In the brains of mice where the transgene was transcribed, expression was neuronal specific, although any neural-restrictive silencing elements are not present in the region of the gene sequenced (Schoenherr and Anderson, 1995). No expression was seen in glial cells or in peripheral tissues (kidney, heart, and liver). In offspring derived from one of the founders (founder A), a virtually correct

pattern was seen. In this line, a large proportion of granule cells in certain folia expressed β-galactosidase (Fig. 7A). However, other folia had far fewer positive cells (Fig. 7B). This inherited mosaic pattern in the cerebellum is reminiscent of those obtained with transgenes incorporating the regulatory regions derived from the Purkinje cell-specific gene *Pcp-2* or the hindbrain-specific gene *engrailed-2*, which, depending on their integration position in the mouse genome, were only highly expressed in posterior Purkinje cells or posterior granule and stellate/basket cells (Vandaele et al., 1991; Logan et al., 1993). Thus, in addition to granule cell-specific enhancers, there might also exist spatial- or compartment-specific enhancers important in defining expression patterns within the adult cerebellum (Logan et al., 1993). Alternatively, the mosaic α6 transgene expression may be connected with the methylation status of the transgene locus (McGowan et al., 1989; Mercer et al., 1991). It is of interest that the 0.5-kb α6 promoter segment in other expressing founder mice gave neuronal-specific expression, e.g., in Purkinje cells and striatal cells, but not in cerebellar granule cells. The chromosomal site of integration of the transgene clearly has a strong influence. One possibility is that the basal promoter elements in the α6 promoter fragment enable the *lacZ* transgene to act as an "enhancer trap" for other neurally expressed genes (Allen et al., 1988).

The α1, β2, γ2, and α6 subunit genes are clustered on human chromosome 5q (Hicks et al., 1993; Russek and Farb, 1994), but it is unknown whether this clustering is related to any coordinate regulation of gene expression, e.g., long-range enhancer elements. However, information within the 0.5-kb proximal region of the α6 subunit gene specifies neuron-specific expression, although other regions of the gene (5′, 3′, or intronic) probably are required to confer the full expression pattern and are needed to insulate the reporter gene from position effects. Within the sequenced region there are five putative homeodomain transcription factor core binding sites, which, in other contexts, have been shown to regulate cell differentiation (Ekker et al., 1994). The CK8 mer sequence identified at position −280 has previously been implicated in keratin-specific gene expression (Blessing et al., 1987). The Sox5/6 site could bind an Sry-related HMG box-containing protein, and such factors have been shown to be present in brain (Connor et al., 1995). In contrast to the α6-subunit gene sequence, the proximal promoter region of the δ gene (which is also highly expressed in granule cells) is GC rich and has multiple Sp1 factor binding sites (Sommer et al., 1990; Motejlek et al., 1994). The brain-specific factor-1 binding sequence, identified as having possible involvement in the regulation of the δ gene and granule cell-specific expression (Motejlek et al., 1994), is not present in the proximal region of the rodent α6 gene. The α6 promoter sequence has no obvious homology with the published proximal promoter sequences of other $GABA_A$ receptor subunit genes that are abundantly expressed in cerebellar granule cells (α1 and β3) but that are also widely expressed in other brain regions (Kirkness and Fraser, 1993; Kang et al., 1994; Bateson et al., 1995).

In summary, by the standard of $GABA_A$ receptors, the α6 subunit gene is relatively small at 14 kb. The gene is transcribed from a TATA-less promoter using multiple start sites, and the proximal 5′ region of the α6 gene can confer neuronal-specific gene expression. These findings provide a foundation for the elucidation of gene-regulatory regions contributing to neuronal subtype-specific gene expression.

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Functional Characterization of the Murine Serotonin Transporter Gene Promoter in Serotonergic Raphe Neurons

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Abstract: We have isolated and characterized the 5′-flanking regulatory region of the murine serotonin 5-HT transporter (5-HTT) gene. A TATA-like motif and several potential binding sites for transcription factors, including two AP1, several AP2 and AP4 binding sites, CCAAT and GC boxes (SP1 binding sites), a nuclear factor-κB, and a cyclic AMP response element-like motif, are present in the 5′-flanking region. A ~2.2-kb fragment (−2,143 to +51 with respect to the transcription start site), which had been fused to the luciferase reporter gene and transiently expressed in a 5-HTT-expressing cell line and in serotonergic raphe neurons derived from embryonic rat brainstem, displayed both constitutive and inducible promoter activity. Functional promoter mapping revealed two clusters of activating elements from bp −82 to −527 and bp −1,001 to −1,937. A cell/neuron-selective silencer element(s) is contained between bp −294 and −527. Our findings suggest that (1) the murine 5-HTT gene promoter is active in serotonergic raphe neurons but significantly repressed in neuronal cells from frontal cortex that do not express 5-HTT, (2) the information contained within ~0.5 kb of the 5′-flanking sequence is sufficient to confer its cell-selective expression, (3) the promoter responds to cyclic AMP- and protein kinase C-dependent induction, and (4) the expression of the 5-HTT is regulated by a combination of positive and negative *cis*-acting elements operating through a basal promoter unit defined by a TATA-like motif. Fusion of the 5-HTT gene promoter unit to a gene of choice may aid its cell-selective expression in transgenic strategies. **Key Words:** Murine serotonin transporter gene—Serotonergic raphe neurons—Basal promoter—*cis*-acting elements—TATA-like motif—Cell-selective expression.
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By modulating the magnitude and duration of postsynaptic receptor-mediated signaling, the serotonin (5-HT) transporter plays a key role in the spatiotemporal fine-tuning of 5-HT neurotransmission (Blakely et al., 1994; Lesch, 1997). Several therapeutic drugs [antidepressants (e.g., fluoxetine)], drugs of abuse [amphetamines, 3,4-methylenedioxymethamphetamine (MDMA, ecstasy)], and potent neurotoxins act on this 5-HT transporter (5-HTT). Investigations using different approaches are increasingly focusing on the physiological consequences of allelic variation in 5-HTT expression and its impact on early brain development and event-related synaptic plasticity. A polymorphism in the 5′ regulatory region of the human 5-HTT gene is associated with anxiety- and depression-related personality traits, and clinical studies suggest that it influences the risk of developing affective disorders, alcohol dependence, late-onset Alzheimer's disease, and autism (Collier et al., 1996; Lesch et al., 1996; Cook et al., 1997; Klauck et al., 1997; Li et al., 1997; Sander et al., 1997).

For the validation of the concept of the 5-HTT gene as a susceptibility locus for emotionality and psychiatric disorders, comparisons across species are gaining momentum. We have recently isolated the gene encoding murine 5-HTT and determined the sequence of all exons including adjacent intronic regions and the 5′-flanking regulatory region (Bengel et al., 1997). The murine 5-HTT gene is composed of 14 exons spanning ~34 kb, and anchored PCR mapped the transcription start site 186 bp upstream of the translation initiation codon. It is noteworthy that the murine 5-HTT gene is located in the vicinity of a quantitative trait locus for alcohol preference in mice with sex-restricted expression and a parent-of-origin effect (Melo et al., 1996). Mice with a targeted disruption of the 5-HTT gene

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Abbreviations used: cAMP, cyclic AMP; CRE, cyclic AMP response element; DMEM, Dulbecco's modified Eagle's minimal essential medium; 5-HT, serotonin; 5-HTT, serotonin transporter; 5-HTTLPR, 5-HTT gene-linked polymorphic region; JAR, human placental choriocarcinoma cells; MDMA, 3,4-methylenedioxymethamphetamine; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

display reduced brain 5-HT concentration and insensitivity to MDMA-induced locomotor activity (Bengel et al., 1998). To further understand transcriptional regulation of the murine 5-HTT gene, we have mapped its 5′-flanking region and examined its function in serotonergic raphe and nonserotonergic neuronal cell cultures derived from embryonic rat brains.

MATERIALS AND METHODS

DNA cloning

A mouse c129 genomic P1 library was screened by PCR targeting exon 2 (Kp1, 5′-TGAGATTCACCAAGGGGA-ACG; Kp2, 5′-CCTCCACCATTCTGGTAGCAT) (Bengel et al., 1997). Clone P1(20) was purified and further characterized by restriction mapping and Southern blot analysis. An ~5-kb *Eco*RI/*Bam*HI fragment of the P1(20) clone containing the 5′-flanking regulatory region (promoter) of the murine 5-HTT gene was then subcloned into the pBK-CMV plasmid (Stratagene), and 3.6 kb of the 5′-flanking sequence of exon 1 was sequenced by using the dideoxynucleotide chain termination (EMBL/GenBank accession number Y08869). Nucleotide sequences were aligned and analyzed using MacVector (IBI) and on-line software (SIGNAL and PROMOTER SCAN).

Promoter plasmid construction

From the ~5-kb *Eco*RI/*Bam*HI insert, a ~2.2-kb *Nhe*I/*Xma*I (N, from bp −2,143 to +51 with respect to the transcription start site) was ligated into the promoterless luciferase (*luc*+) expression vector pGL3 basic (Promega). The following series of deletional mutants of the P-*Nhe*I*luc*+ construct were also examined for their transcriptional activity: P-*Pst*I*luc*+ (P, bp −1,937 to +51), P-*Xho*I*luc*+ (Xh, bp −1,001), P-*Xma*I*luc*+ (Xm, bp −527), P-*Spe*I*luc*+ (Sp, bp −294), and P-*Sac*I*luc*+ (Sa, bp −82). Inserts and insert–vector boundaries were verified by sequence analysis.

Luciferase reporter gene assays

For transient reporter gene expression, human placental choriocarcinoma cells (JAR) and neuronal cell cultures were exposed for 48 h to ~5 μg of plasmid DNA (adjusted for different insert/vector length ratios), complexed with 5 μl of Transfectam lipofectin reagent (Promega). Luciferase gene expression was studied in comparison with the promoterless vector pGL3 basic. Transfection efficiency was assessed by cotransfection with the pSV-βGal. Promoter activity was induced with 100 μ*M* forskolin and 1 μ*M* phorbol 12-myristate 13-acetate (PMA) and determined 24 h after transfection. Cells were harvested in 1 ml of luciferase lysis buffer (Promega). Extracts were assayed for luciferase activity by addition of 20-μl cell lysate samples in 15-s intervals to 100 μl luciferin reagent. Chemiluminescence was counted for 15 s at a constant time (90 s) after reagent mixing in a liquid scintillation spectrometer (Beckman). Luciferase activity was expressed as cpm per microgram of protein. All experiments were done in triplicate.

JAR cell culture

The human placental choriocarcinoma cells (JAR; American Type Culture Collection HTB 144) express the human 5-HTT (Cool et al., 1991; Ramamoorthy et al., 1993). These cells were grown in RPMI 1640 supplemented with 10% newborn calf serum at 37°C in a humidified atmosphere at 5% CO_2. Before transfection cells were washed twice with sterile phosphate-buffered saline (PBS), trypsinized, and 2 × 10^5 cells were grown in 35-mm plates.

Neuronal cell cultures

Fetal rats (embryonic day 15) were obtained from time-pregnant Sprague–Dawley rats and dissociated as previously described (Wichems et al., 1995). In brief, regions of the frontal cortex and the rostral portion of the rhombencephalon extending from the rhombencephalic isthmus to the pontine flexure were dissected and placed in sterile Dulbecco's modified Eagle's minimal essential medium (DMEM) in 35-mm Petri dishes. This region contains the major 5-HT-containing cell groups located within the dorsal and medial raphe nuclei (Dahlstrom and Fuxe, 1964; Konig et al., 1988). Tissues were rinsed twice and mechanically dispersed. The cells were quantified and plated at 3 × 10^5 cells per Lab Tek slide well or 1.4 × 10^6 cells per 35-mm well. All culture surfaces were precoated overnight with poly-L-lysine (100 μg/ml). The plating media consisted of DMEM (4.5 mg/L glucose, no pyruvate)/Ham's F12 (3:1) supplemented with 10% fetal calf serum, 10% horse serum, 2 m*M* glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. After 24 h, this medium was removed and replaced with medium containing 2% serum. The 2% serum medium remained on the culture for 24 h and was then replaced with serum-free medium (N2 medium). The cultures were maintained in an atmosphere of 5% CO_2 at 37°C and transfected after 5 days.

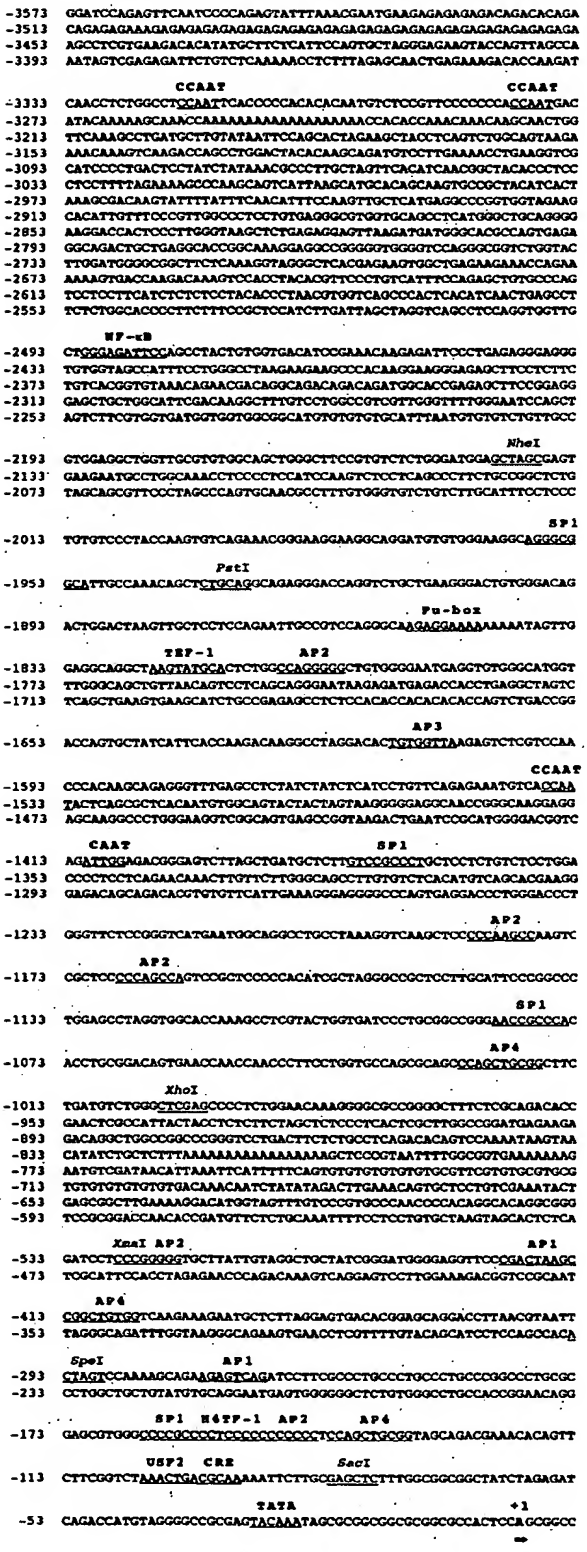
Immunocytochemical procedures

Cell cultures derived from embryonic rat brainstem and frontal cortex were fixed with 4% paraformaldehyde on Lab Tek slides for 30 min and rinsed with PBS. After incubation for 20 min with normal goat serum, the cells were incubated for 24 h with the primary antibodies specific for 5-HT (1:40,000; IncStar) and neuron-specific enolase (NSE, 1:15,000; Polysciences). Tissues were processed according to the Vectastain protocol (Vector Laboratories), using diaminobenzidine as the chromogene.

RESULTS

The sequence of the 5′-flanking region of the murine 5-HTT gene is shown in Fig. 1. A single transcription start site 186 bp upstream from the translation initiation codon located in exon 2 had previously been detected by PCR-assisted primer extension. 5′ of the transcription start site, a TATA-like motif (TACAAA; bp −26 with respect to the transcription initiation site), two AP1 binding sites, several AP2 and AP4 binding sites, CCAAT and GC boxes (SP1 binding sites), a nuclear factor-κB (NF-κB), and a cyclic AMP (cAMP) response element (CRE)-like motif were identified (Table 1). Figures 1 and 2A depict known *cis*-acting elements that appear to be assembled in two major clusters from bp −82 to −527 and from bp −1,001 to −1,937.

Transfection experiments in human JAR cells demonstrated moderate constitutive promoter activity of the 2.2-kb P-*Nhe*I*luc*+ construct, whereas the



sequence information contained within the P-*SpeIluc*+ and P-*SacIluc*+ deletional mutants was not sufficient to confer promoter activity in JAR cells (Fig. 2C). Cotransfection experiments with the plasmid pSV-βGal demonstrated a variance of transfection efficiency of <5%. Transcriptional activity of the 2.2-kb P-*NheIluc*+ construct was also studied in serotonergic and nonserotonergic neuronal cell cultures derived from embryonic rat brainstem and frontal cortex, respectively. Immunocytochemical procedures defined most cells as embryonic glial cells; serotonergic cells were absent in the cultures obtained from the frontal cortex, whereas the density of serotonergic cells in the cultures obtained from the brainstem was ~10% (Fig. 3). Robust promoter activity could be demonstrated in serotonergic raphe cell cultures, but in nonserotonergic cell cultures derived from cortical regions this activity was significantly repressed (Fig. 2C).

To determine the *cis*-acting elements required for inducible and cell-selective promoter activity, a series of nested deletions of the P-*NheIluc*+ construct were made (Fig. 2A and B). Serial 5′ deletions from bp −2,143 to −82 (with respect to the transcriptional start) of the promoter construct resulted in a gradual decrease of constitutive transcriptional activity in human JAR and embryonic rat raphe cells (Fig. 2C). Transcriptional activity in embryonic rat raphe cells was usually two- to threefold higher than in human JAR cells. Deletion experiments also demonstrated that core promoter sequences, including a 5-HTT-expressing cell/neuron-selective silencing element(s), are contained within ~0.5 kb of the transcription start site. In nonserotonergic cortical cells a substantial attenuation of promoter activity was detected in the P-*XmaIluc*+ construct, compared with the P-*SpeIluc*+ construct, which essentially persisted in all constructs with deletions located further upstream. This indicates that a major transcriptional repressor element is likely to be located between bp −294 and −527.

Forskolin and PMA induced 5-HTT promoter-driven luciferase gene expression in transiently transfected serotonergic raphe neurons (Fig. 4). cAMP- and protein kinase C (PKC)-dependent activation of the promoter was ~20–50% increased above basal activity of the P-*NheIluc*+ construct. Studies with the deletional mutants showed the highest relative increment of inducible activity in the

FIG. 1. Sequence of 5′-flanking region of the murine 5-HTT gene (EMBL/GenBank accession number Y08869). The transcription start site is represented by an arrow. The core promoter unit is defined by a TATA-like motif delineated by an AT cluster (TACAAA) 26 bp upstream of the transcription start site. Numbering of nucleotides is shown on the left side, the transcription start site is nucleotide +1 of exon 1. Potential binding sites for transcription factors are underlined. Common restriction enzyme recognition sites are indicated by dotted underline.

TABLE 1. *Promoter sequence motifs with possible regulatory function in murine 5-HTT gene expression*

Element	Mouse sequences	Consensus sequence	Position	Inducer/repressor
AP1	aGAGTCAg	TGAG/CTC/AA	−270	cAMP, PMA, NGF, TGF-β, IL-2
	cGACTAAg		−476	
AP2	CCCCCCCC	CCCA/CNG/CG/CG/C	−144	cAMP, PMA; also interacts with SP1
	CCCGGGGG		−520	
	CCCAGCCa		−1,160	
	CCCAAGCC		−1,179	
	CCaGGGGG		−1,801	
AP3	TGTGGTTA	TGTGGA/TA/TA/T	−1,608	
AP4	CCAGCTGCGG	C/TCAGCTGC/TGG	−133	
	CCgGCTGTGG		−405	
	CCAGCTGCGG		−598	
SP1	CCCCGCCCC	G/TG/AGGCG/TG/AG/AG/T	−156	Unspecific, increases number of productive transcription complexes
	AaCCGCCCA		−1,076	
	gTCCGCCCa		−1,372	
	aGGGCGGca		−1,951	
CCAAT box	ATTGG	CCAAT	−1,407	Multiple interactions
	CCAAT		−1,533	
	CCAAT		−3,277	
	CCAAT		−3,316	
NF-κB	GGGAgaTTCC	GGGAA/CTNT/CCC	−2,482	cAMP, PMA, IL-1, TNF-α
Pu box	AGAGGAAaa	AGAGGAACT	−1,845	
TEF-1	AAGTATGCA	AAGT/CATGCA	−1,815	
H4TF-1	CCCTCCCCC	GGGGGAGGG	−150	
USF2	AAACTGACGCA	AAACTGACGCA	−94	Stabilizes the TFIID preinitiation complex
CRE	TGACGCAA	TGACGC/TC/AG/A	−93	cAMP, Ca^{2+}

P-*XmaIluc*+ (~35–45% increase above the basal activity) and P-*PstIluc*+ (~50–90%) constructs, which encompass one or two clusters of *cis*-acting elements, respectively. Each of these clusters contain several AP1 and AP2 binding sites. In addition, a CRE-like element is located at bp −95. However, when this putative CRE element was induced with forskolin separately from other enhancer elements within the P-*SpeIluc*+ construct, it showed only a negligible effect on transcriptional activity.

DISCUSSION

The 5′-flanking region of the murine 5-HTT gene has been isolated, mapped, and characterized functionally. Although 5′-RACE (rapid amplification of cDNA 5′ ends) analysis had demonstrated a single transcription start site 186 bp upstream of the translation initiation codon, no classic TATA-box was found. However, both the murine and human transcription start sites are associated with a perfectly conserved TA-rich sequence, TACAAA, at the identical upstream distance bp −26 (Bengel et al., 1997; see Fig. 5).

Considerable variations in the classic TATA/ATA motifs have been reported to be functional in promoter elements of many genes expressed in neuronal and nonneuronal cells, including the following variants AATTAA, TTAA, TTTAAA, ACATAA, TTAAT, and AAATAT (Evans et al., 1988; Hobson et al., 1988; Sibley et al., 1989; Bloem et al., 1993; Pu et al., 1993). Comparison of the murine and human 5-HTT 5′-flanking region revealed ~82% sequence conservation in the GC-rich 130 bp immediately upstream of the transcription start site. Alignment of the murine and human 5′-flanking sequence 3.5 kb upstream displayed no further striking matches.

We have shown that a considerable number of potential binding sites for transcription factors organized in two major clusters are present in the 5′-flanking region. The role of these binding motifs for *trans*-acting elements in the regulation of murine 5-HTT gene expression has been established by transfection studies in human and rodent cell cultures by using reporter gene fusion constructs of 5-HTT gene 5′-flanking sequences. It is interesting that in human JAR cells, promoter activity was considerably lower than in rat raphe neurons. Although the location of the transcription start site and several transcription factor binding motifs in the mouse and human 5-HTT gene 5′ regulatory region is similar (Heils et al., 1995), this

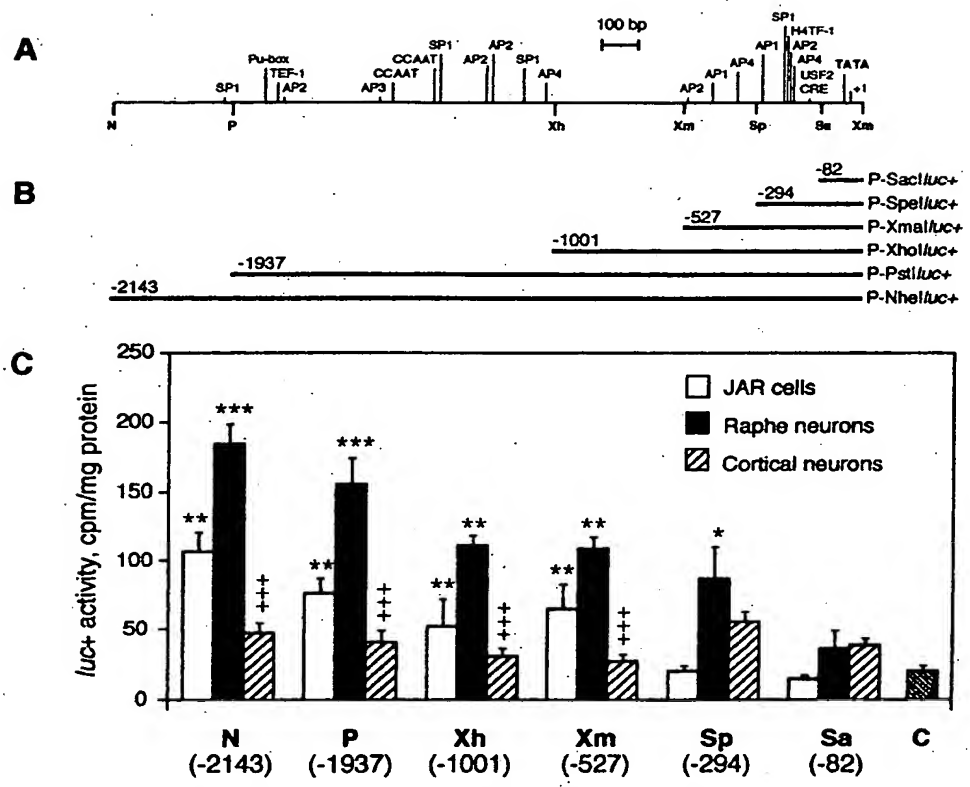


FIG. 2. **A:** Map of the 5′-flanking region of the murine 5-HTT gene. Potential binding sites for transcription factors and restriction enzyme recognition sites used for the 5′ deletions are marked. **B:** Serial 5′ deletions of the murine 5-HTT. Deletional mutants of the P-*Nhe*I*luc*+ construct: P-*Pst*I*luc*+ (P, bp −1,937 to +51), P-*Xho*I*luc*+ (Xh, bp −1,001), P-*Xma*I*luc*+ (Xm, bp −527), P-*Spe*I*luc*+ (Sp, bp −294), and P-*Sac*I*luc*+ (Sa, bp −82). **C:** Cell-selective expression and effects of 5′ deletions on the constitutive transcriptional activity of the murine 5-HTT gene promoter. The role of transcription factor motifs in the regulation of 5-HTT gene expression was established by transfection of human and rodent cells using luciferase reporter gene (*luc*+) fusion constructs of serially deleted 5-HTT 5′-flanking sequences. Luciferase gene expression was studied in comparison with the promoterless vector pGL3 basic (C, control). Transfection efficiency was assessed by cotransfection with the pSV-β-Gal. P-*Sac*I*luc*+ (Sa) versus Sp, Xm, Xh, P, and N for each cell type: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; cortical versus raphe neurons: $^{+++}p < 0.001$, one-way ANOVA followed by Fisher's PLSD test.

result implies differences in the cooperativity of *cis*- and *trans*-acting elements between mouse and human 5-HTT promoters.

Functional promoter mapping revealed both constitutive and forskolin/PMA-induced promoter activity and a role for negative attenuating elements and positive elements in the transcriptional regulation of the murine 5-HTT gene. The information contained within ~0.5 kb of the 5′-flanking sequence was sufficient to confer cell-selective expression in 5-HTT-expressing human JAR cells and rat raphe neurons, with significantly repressed 5-HTT promoter activity in nonserotonergic neuronal cells derived from the rat frontal cortex. Although a major transcriptional repressor appears to be located between bp −294 and −527, we did not find any consensus sequences in this region that are similar to recently characterized neuron-selective silencer elements, such as the rat sodium channel gene (Maue et al., 1990) or the α2 subunit of the rat nicotinic acetylcholine receptor gene (Bessis et al., 1993).

Our results demonstrated that the murine 5-HTT promoter is induced by cAMP- and PKC-dependent mechanisms. Studies with deletional mutants indicated that regulation of forskolin and PMA-inducible promoter activity depends on multiple *cis*-acting elements (Faisst and Meyer, 1992). The somewhat lower cAMP- and PKC-dependent activation of the promoter in the mouse gene (about twofold), compared with the four- to fivefold inducible activation of the human 5-HTT gene promoter (Heils et al., 1996), may be explained by the ratio of 5-HT-specific to 5-HT-negative cells in the embryonic raphe cell culture of 1:9 and a possible inhibitory cross-

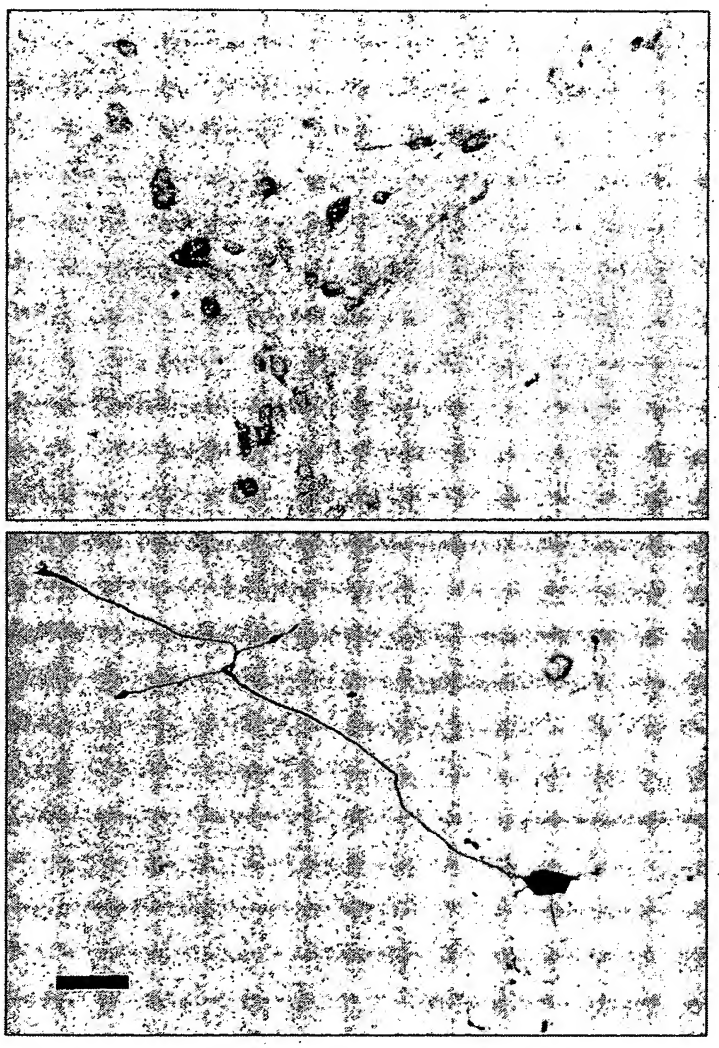


FIG. 3. Immunocytochemistry of cell cultures derived from embryonic rat brainstem. Photomicrographs of neuronal and glial cells incubated with antibodies against neuron-specific enolase (**top**) and with antibodies specific for 5-HT (**bottom**). The density of serotonergic cells in the cultures obtained from the brainstem was ~10%. Bar, 25 μm.

talk between the two cell populations. Studies with the deletional mutants indicated that regulation of cAMP- and PKC-induced promoter activity depends on the two clusters of *cis*-acting elements containing several AP1 and AP2 binding sites and a putative CRE element located at bp −95. It should be noted, however, that this CRE-like element within the P-*Spelluc*+ construct demonstrated only a negligible effect on transcriptional activity when induced independently from other enhancer elements.

Forskolin and PMA-induced enhancement of 5-HT uptake, inhibitor binding, and mRNA concentrations indicative of cAMP-dependent transcriptional activation of the 5-HTT gene expression have been reported previously in JAR cells, although PKC-dependent phosphorylation of the 5-HTT protein may counteract the genomic effects (Cool et al., 1991; Ramamoorthy et al., 1993, 1995). An 8-bp palindromic CRE-like enhancer sequence is present at bp −93 in the murine 5-HTT gene (at bp −94 in the human 5-HTT gene), which is in agreement with the finding that CRE elements are close to the transcription start site (frequently within 150 bp). Our results, however, suggest that additional enhancer elements, such as AP1, AP2, or NF-κB binding sites, contribute to cAMP-induced promoter activation. AP1 and AP2 binding sites are commonly activated by cAMP-inducible immediate-early gene products including transcription factors of the c-*fos*/c-*jun* family. Moreover, a large number of genomic activation domains, whose function depends on the interaction with classic transcription factors, have been characterized in

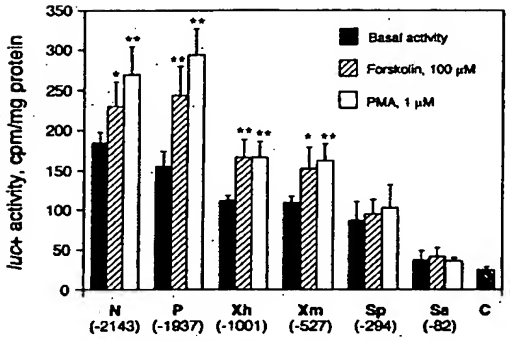


FIG. 4. Forskolin- and PMA-induced transcriptional activity of the murine 5-HTT gene promoter. Serotonergic cell cultures derived from the rat brain raphe complex were transfected with 5 μg of the P-*Nhe*I*luc*+ construct or deletional mutants, followed by treatment with forskolin (100 μ*M*) or PMA (1 μ*M*). Luciferase gene expression was studied in comparison with the promoterless vector pGL3 basic (C, control). Basal versus forskolin- or PMA-induced activity: $^{*}p < 0.05$, $^{**}p < 0.01$; one-way ANOVA followed by Fisher's PLSD test.

recent years. Among these, the USF2 binding site located at bp −94 may interact with a family of basic helix–loop–helix transcription factors that stabilize the formation of the TFIID preinitiation complex (Luo and Sawadogo, 1996). This and other interactions are commonly involved in transcriptional initiation as well as in the regulation of tissue-selective and developmentally regulated genes.

In the human 5-HTT gene 5′-flanking regulatory region, we recently reported the presence of a unique polymorphic repetitive element, the 5-HTT gene-linked polymorphic region (5-HTTLPR) (Heils et al., 1996). This 5-HTTLPR contains activating and repressing elements and confers allelic variation of functional 5-HTT expression in humans. It is associated with anxiety- and depression-related personality traits (Lesch et al., 1996) and may influence the risk of developing affective disorders, alcohol dependence, late-onset Alzheimer's disease, and autism (Collier et al., 1996; Cook et al., 1997; Klauck et al., 1997; Li et al., 1997; Sander et al., 1997). It is noteworthy that this or a similar polymorphic repetitive element was not detected in the 5′-flanking sequence of the mouse gene, thus further supporting the view of significant interspecies variation in the regulation of 5-HTT gene expression.

Several potential binding sites for transcription factors, including AP1, AP2, AP4, SP1, and as yet unknown silencer elements, are likely to confer cell-selective regulation of gene transcription (Levine and Manley, 1989; Struhl, 1991). This notion is supported by an increasing body of evidence showing that several intracellular signal transduction pathways converge on the transcriptional apparatus of the 5-HTT gene, and that expression is regulated by cAMP, PKC, and tyrosine kinase-dependent mechanisms (Cool et al., 1991; Ramamoorthy et al., 1993; Heils et al., 1995). However, the functionality of these transcription factor motifs and silencer elements in the regulation of mouse 5-HTT gene expression requires further studies using gel-shift assays, footprinting, and cotransfection of 5-HTT gene promoter constructs with transcription factor expression vectors.

One of the potential applications of the murine 5-HTT transcription regulatory sequence is to confer cell selectivity in gene transfer strategies. Fusion of the 5-HTT gene promoter unit to a gene of choice is likely to ensure that the transferred gene is transcribed only in serotonergic raphe neurons. Although the 5-HTT gene promoter may be studied for cell-selective gene expression by transfection into cells in culture, confirmation of 5-HT cell-selective expression of a transferred gene by generation of transgenic mice is ultimately required, because the promoter/transgene construct is incorporated into the genome and is therefore present in all cell types. As a consequence, cell type-selective promoter activity has physiological relevance only when the introduced transgene is expressed in a tissue-restricted pattern independent of the site of integration.

In conclusion, the findings indicate that (1) the murine 5-HTT gene promoter is active in human JAR cells and serotonergic cell cultures derived from embryonic rat brainstem, but characteristically repressed in nonserotonergic neuronal cells from frontal cortex, (2) the information contained within ~0.5 kb of 5′-flanking sequence is sufficient to confer its cell-selective expression, (3) the promoter responds to cAMP- and PKC-dependent induction, and (4) the expression of the 5-HTT is regulated by a combination of positive and negative *cis*-acting elements operating through a basal promoter unit defined by a TATA-like motif. Fusion of the 5-HTT gene promoter unit to similar or unrelated genes may aid their selective expression in the central 5-HT system and thus ease novel transgenic research strategies.

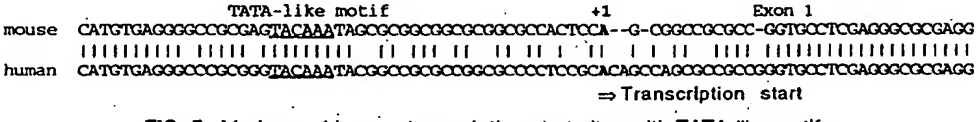


FIG. 5. Murine and human transcription start sites with TATA-like motifs.

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EXHIBIT C

DESCRIPTION

MODEL ANIMALS FOR VISUALIZATION OF NEURAL PATHWAYS

Filed of the Invention

The present invention relates to transgenic animals into which a gene encoding a trans-synaptic tracer protein is introduced so as to direct specific expression in particular types of neurons, a method for screening neuromimetic substances using the transgenic animals, and a neuromimetic substance obtainable by the screening method.

Prior Art

The brain with its various functions including learning, memory, multisensory recognition and integration, motor development and control, as well as emotion is composed of complex, but well-ordered neural networks. To study brain structure and function, there is a need to understand the molecular mechanisms for formation, maintenance and plasticity of neural pathways. In particular, it is undoubtedly the touchstone of studies in these various areas of neuroscience to elucidate how neurons extend their axons in the correct direction, how they recognize target cells, how they form synapses, how they form and maintain neural networks, and how they further plastically change the formed neural pathways as needed.

A variety of plant lectins have been conventionally used as trans-synaptic tracers in neuroanatomical studies on neuronal connectivity. In particular, wheat germ agglutinin (WGA) has been most efficiently transferred from primary neurons to secondary neurons across synapses, thereby exhibiting its usefulness in any neural systems. In the visual system, for example, WGA injected into one eye is taken up by ganglion cells of the retina and then transported through optic nerves to the lateral geniculate nucleus of the thalamus, where WGA is trans-synaptically transferred to thalamic secondary neurons, resulting in a WGA-labeled visual cortical area which is the projection site of the thalamic secondary neurons. In this way, the ocular

dominance columns can be visualized. Thus, the technique using WGA as a tracer is very useful and powerful, and has greatly contributed to the development of neuroscience.

However, the above conventional tracing technique using WGA does not allow selective visualization of functional neural pathways through a particular group of neurons because WGA was taken up by all the cells surrounding the site of WGA injection. In addition, other problems have also been pointed out, for example, serious immune responses induced in a WGA-injected animal due to the recognition of WGA as a foreign substance.

Problems to be solved by the Invention

The tracing technique using WGA is very useful for studying functional connectivity patterns between neurons, but it also involves the various problems mentioned above. The object of the present invention is to overcome these problems.

Means for solving the Problems

Our research efforts were directed to overcoming the above problems, and we have found that these problems can be overcome by using a transgenic animal into which a gene encoding a trans-synaptic tracer protein is introduced so as to direct specific expression in particular neurons, thereby finally completing the invention.

Thus, the present invention provides transgenic animals into which a gene encoding a trans-synaptic tracer protein is introduced so as to direct specific expression in particular neurons.

The present invention also provides a method for screening neuromimetic substances, which comprises administering a test substance to the transgenic animal mentioned above, and selecting a neuromimetic substance from among the test substances by using as an indicator the trans-synaptic tracer protein expressed in the animal's neurons.

The present invention further provides a neuromimetic substance obtainable by

the screening method mentioned above.

This specification includes part or all of the contents as disclosed in the specification and/or drawings of Japanese Patent Application No. 10-232817 which is a priority document of the present application.

Disclosure of the Invention

The present invention will now be described in more detail.

The transgenic animal of the present invention is characterized in that a gene encoding a trans-synaptic tracer protein is introduced so as to direct specific expression in particular neurons.

In addition to WGA mentioned above, examples of the trans-synaptic tracer protein include, but are not limited to, Concanavalin A agglutinin (ConA), Pisum Sativum agglutinin (PSA), Lens Culinaris agglutinin (LCA) and the like. To express a gene encoding a trans-synaptic tracer protein in particular neurons, a promoter specific to the particular neurons may be connected upstream of the gene, but any other technique can be used for this purpose. The promoter specific to particular neurons includes, but is not limited to, cerebellar Purkinje cell-specific L7 promoter, olfactory receptor cell-specific OMP promoter and the like. As used in the transgenic animals of the present invention, the term "specific" or "specifically" means that the trans-synaptic tracer protein is sufficiently expressed to distinguish particular neurons from other cells when it is visualized with an enzyme-labeled antibody etc., but it does not necessarily mean that no trans-synaptic tracer protein gene is expressed in any other cell.

A wild-type gene encoding the trans-synaptic tracer protein may be used without any further modification. For the WGA gene, the modified gene that lacks a C-terminal propeptide-coding segment may be preferably used for the reason described below.

Any animal having neural pathways may be used in the present invention.

The transgenic animal of the present invention may be constructed as follows. A fragment containing the promoter specific to particular neurons and a fragment

containing the trans-synaptic tracer protein gene may be amplified by PCR, respectively, and these amplified fragments may then be inserted into an existing vector for recombination. The resulting recombinant vector may be injected into fertilized eggs or embryos from recipient animals. A transgenic animal having the trans-synaptic tracer protein gene may be selected out of the resulting animals. For the L7 promoter, the fragment containing the promoter specific to particular neurons may be obtained by preparing primers that can amplify a region upstream from the initiation codon of the nucleotide sequence shown in SEQ ID NO: 2; amplifying a part of the promoter region by PCR using mouse genomic DNA as a template; and then screening a mouse genomic DNA library (e.g., commercially available mouse Genomic DNA Library SC945301 (Stratagene)) using the amplified PCR product as a probe. For the OMP promoter, such a fragment may be obtained by preparing a primer that can amplify a region upstream from the initiation codon of the nucleotide sequence shown in SEQ ID NO: 3; and carrying out PCR using mouse genomic DNA as a template. For the WGA gene, the fragment containing the trans-synaptic tracer protein gene may be obtained by preparing a primer that can amplify a coding-region of the nucleotide sequence shown in SEQ ID NO: 1; and carrying out PCR using wheat germ cDNA as a template.

The transgenic animal of the present invention is useful for the elucidation of causes for various neurogenic diseases and the establishment of medical treatment for these diseases. For example, the transgenic animal of the present invention may be crossed with an animal model for diseases resulting from abnormal neural pathways or with a spontaneously mutated animal model. The resulting animal may then be used to analyze the abnormal neural pathways responsible for the diseases or compensatory pathways induced by the diseases. The transgenic animal of the present invention may also be used to create an artificial pathological model for Parkinson's disease, ischaemia, head injury or various mental diseases for the analysis of injured pathways or compensatory pathways. Further, the transgenic animal of the present invention developing pathological conditions may be administered with various drugs in order to assess the potency of the administered drugs, i.e., their ability to restore injured

pathways or to form compensatory pathways, by using trans-synaptic tracer protein as an indicator.

In addition, the transgenic animal's tissues expressing the trans-synaptic tracer protein may be primarily cultured to create cultured neurons expressing the trans-synaptic tracer protein, which may then be used for the screening of drugs that affect cell survival and maintenance, dendrite extension, synapse formation, various enzymatic activities, and/or neurotransmitter production.

The neuromimetic substance of the present invention may be obtained according to the above screening methods, for example, by screening test substances such as peptides, proteins, non-peptide compounds, synthetic compounds, fermented products from microorganisms, marine organism extracts, plant extracts, cell extracts, or animal tissue extracts. These test substances may be novel or known compounds.

The present invention will be further described in the following example. The example is provided for illustrative purposes only, and is not intended to limit the scope of the invention.

Examples

Example 1: Construction of a vector expressing WGA

A wild-type WGA cDNA insert (1.0 kb) excised from pWGA-D (Smith, J. J. & Raikhel, N.V., Plant Mol. Biol. 13, 601-603 (1989)) was blunt-ended, followed by addition of a BstX I site. This insert was subcloned into a BstX I site of a mammalian expression vector pEF-BOS (Mizushima, S. & Nagata, S., Nucl. Acids Res. 18, 5322 (1990)) to construct a plasmid pEF-WGA. Mouse nueroblastoma N2a cells were transfected with this plasmid using Lipofectamine and Opti-MEM (Gibco/BRL). After 48 hours of transfection, the cells were tested for the presence of expressed WGA by Western blotting using anti-WGA antibody. As shown in Figure 2, WGA was detected (lane 2, MW 24kD), but it had a significantly larger size than that of an authentic WGA (lane 4, MW 18 kD).

Since the plant WGA has a C-terminal propeptide (15 amino acid residues)

which is involved in selective delivery of the lectin into vacuoles (Broadwell, R. D. & Balin, B. J., J. Comp. Neurol. 242, 632-650 (1985)), the difference in molecular weight was thought to be caused by the absence of C-terminal propeptide-processing mechanism in animal cells. Thus, we decided to construct a plasmid containing a DNA segment encoding a truncated WGA which lacks the C-terminal propeptide.

A plasmid pEF-tWGA containing the truncated WGA-coding cDNA (Figure 1(2)) was constructed from the plasmid pEF-WGA by replacing a codon GTC (valine 198) in the wild-type WGA-coding DNA (Figure 1(1)) with an opal stop codon TGA by PCR mutagenesis using not-completely complementary primers. The N2a cells were transfected with this plasmid pEF-tWGA and then tested for the presence of expressed WGA by Western blotting, thereby detecting the truncated WGA having the same size as the authentic WGA (Figure 2, lane 3). In addition, the amount of the truncated WGA produced was significantly larger than that of the wild-type WGA. Thus, we decided to use this truncated WGA-coding DNA in all the following experiments.

The N2a cells transfected with pEF-tWGA were treated with anti-WGA polyclonal antibody and Cy3 anti-rabbit antibody IgG (Jackson) (10 μg/ml, Sigma), followed by observation using a confocal laser scanning microscopy system (Bio-Rad MRC-600) equipped with Zeiss Axiophot F1 microscope (Figure 3). As shown in Figure 3, the truncated WGA strongly bound to the intracellular granule-like structures of N2a cells.

Example 2: Construction of a pL7-tWGA-introduced mouse

A mouse L7 promoter region (3.5 kb) was amplified from Pcp2-z06 plasmid (Vandaele, S. et al., Genus Dev. 5, 1136-1148 (1991)). L7 (Pcp2) gene promoter has been analyzed in detail (Oberdick, J. et al., Neuron 1, 367-376 (1988); Oberdick, J. et al., Science 248, 223-226 (1990); Oberdick, J. et al., Neuron 10, 1007-1018 (1993); Vandaele, S. et al., Genus Dev. 5, 1136-1148 (1991)), and used for cerebellar Purkinje cell-specific expression of foreign genes (Feddersen, R. M. et al., Neuron 9, 955-966 (1992); Burright, E. N. et al., Cell 82, 937-948 (1995)). This amplified fragment was

subcloned into a blunt-ended BamH I site of pBstN vector, which contains human β-globin gene introns and SV 40 polyadenylation signal. A tWGA cDNA sequence (0.6 kb) excised from pEF-tWGA was ligated to a blunt-ended EcoR I site of the pBstN vector to construct a plasmid pL7-tWGA for cerebellar Purkinje cell-specific expression of the truncated WGA (Figure 1(3)).

The purified pL7-tWGA was injected into the male pronucleus of fertilized eggs from FVB/N mice (CLEA Japan), mainly according to the procedures described by Nohmi, T. et al., Environment. Mol. Mutagenesis 28, 465-470 (1996). The pL7-tWGA-injected eggs were cultured and transferred into the oviduct of ICR pseudopregnant recipients (CLEA Japan). Tail samples taken for DNA analysis were screened for the integrated transgene by PCR and Southern analysis, thereby obtaining a transgenic mouse having the full-length transgene.

The presence of expressed WGA mRNA and protein in this transgenic animal's brain was determined by in situ hybridization and immunohistochemistry, respectively.

In situ hybridization was carried out according to the procedures described by Yoshihara, Y. et al., J. Neurosci. 17, 5830-5842 (1997) as follows.

Sections (50 μm) of an adult mouse brain perfused with paraformaldehyde were treated with proteinase K (10 μg/ml at 25 °C for 30 min), acetylated, dehydrated, and then air-dried. An antisense riboprobe for WGA (540 nucleotides in length) was prepared using ^{35}S-UTP (Amersham) and an RNA transcription kit (Stratagene). The sections were hybridized overnight with the above antisense riboprobe (1×10^6 cpm/ml) in a humidified chamber at 56 °C. After hybridization, the sections were washed with 4 × SSC, treated with RNase A (10 μg/ml at 37 °C for 30 min), washed with 0.05 × SSC, dehydrated with ethanol, and then exposed to βmax X-ray film (Amersham).

Also, immunohistochemistry was carried out as follows.

Sections (50 μm) of a mouse brain perfused with paraformaldehyde were cut with a sliding microtome, pre-treated with 0.3% H_2O_2, blocked, and then incubated for 2 to 24 hours at room temperature with anti-WGA polyclonal antibody (3 μg/ml, Sigma)

which had been absorbed with 1% acetone powder of mouse brain. The sections were then incubated either with biotin anti-rabbit IgG (Zymed), followed by a Vectastain ABC elite kit (Vector), or with horseradish peroxidase anti-rabbit IgG (Jackson). The generated signals were visualized through the Ni^{2+}-enhanced diaminobenzidine/peroxide reaction for analysis using a transmission microscopy system.

Figure 4 (1) shows WGA mRNA detection in a section including the whole brain tissue. Figure 4 (2) shows WGA protein detection in an adjacent section to the section shown in Figure 4 (1). As shown in both figures, WGA mRNA detection is limited to the Purkinje cells, while the WGA protein is expressed not only in the Purkinje cells, but also in other cells anatomically and functionally associated with the Purkinje cells. Figure 5 shows WGA protein detection in a section including the cerebellum, indicating that the WGA protein is expressed in the deep cerebellar nuclei (dentate, fastigial, interposed) and the vestibular nucleus, as well as the Purkinje cells.

Axons of the Purkinje cells form synapses with neurons in the deep cerebellar nuclei (Ito, M., The cerebellum and neural control., New York, Raven Press (1984); Altman, J. & Bayer, S.A., Development of the cerebellar system: in relation to its evolution, structure, and functions., Boca Raton, Florida, CRC Press (1996)). Since some Purkinje cells directly project to the vestibular nucleus, secondary neurons are also present in the vestibular nucleus. In view of the foregoing, WGA is thought to be transported to secondary and tertiary neurons of the Purkinje cells.

Double immunofluorescence labeling was used to determine the presence of Purkinje cells and expressed WGA protein in the cerebellum of the transgenic mouse transformed with pL7-tWGA. The Purkinje cells were detected by anti-calbindin antibody (Sigma) and FITC anti-mouse IgG (Cappel). Calbindin is specifically found in the Purkinje cells. The WGA protein was detected by anti-WGA antibody and Cy3 anti-rabbit IgG (Jackson). Figures 6 (1) and (2) show WGA protein and Calbindin in the deep cerebellar nuclei, respectively. Figure 6 (3) shows the detection of both. These figures indicate the trans-synaptic transfer of WGA protein from axon termini of the Purkinje cells to neurons in the deep cerebellar nuclei.

Immunohistochemistry with anti-WGA antibody was used to determine the presence of expressed WGA protein in brain parts other than the cerebellum of the transgenic mouse transformed with the plasmid pL7-tWGA. Figures 7 (1) and (2) show WGA protein detection in a section including the thalamic ventrolateral nucleus and in a section including the red nucleus, respectively. Figures 7 (3) and (4) show magnified views of Figures 7 (1) and (2), respectively. Figures 7 (5) to (8) show WGA protein detection in a section including the superior colliculus, in a section including the gigant cellular reticular nucleus, in a section including the vestibular nucleus, and in a section including the inferior olivary nucleus, respectively. These figures indicate that the WGA protein is detected in any of the thalamic ventrolateral nucleus, red nucleus, superior colliculus, gigant cellular reticular nucleus, vestibular nucleus and inferior olivary nucleus. All of these neurons form synapses with axons from the deep cerebellar nuclei, and correspond to tertiary neurons of the Purkinje cells.

Example 3: Construction of a pOMP-tWGA-introduced mouse

An OMP promoter region (0.9 kb; Buiakova, O. I. et al., Genomics 20, 452-462 (1994)) was amplified by PCR from mouse genomic DNA and subcloned into a blunt-ended BamH I site of pBstN vector (Figure 1 (4)). A tWGA cDNA sequence was inserted downstream of the OMP promoter region to obtain a plasmid pOMP-tWGA. This plasmid pOMP-tWGA was used to construct a transgenic mouse, as described in Example 2.

The presence of expressed WGA protein in the vomeronasal organ of the above transgenic mouse was determined by an immunofluorescence labeling technique using anti-WGA antibody and Cy3 anti-rabbit IgG. Figures 9 (1) and (2) show WGA protein detection in a section including the vomeronasal organ and a magnified view thereof, respectively, indicating that the WGA protein is highly expressed in the vomeronasal epithelium and nerve bundles thereof.

Double immunofluorescence labeling was used to determine the presence of axons and expressed WGA protein in the vomeronasal organ of the transgenic mouse

transformed with pOMP-tWGA. The axons were detected by anti-NCAM antibody and FITC anti-mouse IgG (Cappel). NCAM is specifically found in the axons. The WGA protein was detected by anti-WGA antibody and Cy3 anti-rabbit IgG (Jackson). Figures 10 (1) and (2) show WGA protein detection and axon detection, respectively. Figure 10 (3) shows the detection of both. These figures indicate that NCAM is evenly expressed in the olfactory and vomeronasal nerves, while the WGA protein is highly expressed in the vomeronasal nerves, in particular.

Immunohistochemistry was used to determine the presence of expressed WGA protein in the brain of the transgenic mouse transformed with pOMP-tWGA.

Figure 11 shows WGA protein detection in a section including the whole brain, indicating that the WGA protein is highly expressed in the accessory olfactory bulb.

Figures 12 (1) and (2) show WGA protein detection in a section including the accessory olfactory bulb, indicating that the WGA protein is expressed not only in vomeronasal axon termini of the glomerulus, but also in external plexiform layer and granule cell layer thereof.

Figures 13 (1), (2) and (3) show WGA protein detection in a section including the lateral olfactory tract, indicating that the WGA protein is also expressed in mitral/ tufted cell axons of the lateral olfactory tract, which corresponds to axons of secondary neurons.

Figures 14 (1) and (4) show WGA protein detection in a section including the medial amygdaloid nucleus. Figures 14 (2) and (3) show WGA protein detection in a section including the posteromedial cortical amygdaloid nucleus and in a section including the bed nucleus of stria terminalis, respectively. These figures indicate that the WGA protein is expressed in any of the medial amygdaloid nucleus, posteromedial cortical amygdaloid nucleus, and bed nucleus of stria terminalis, which correspond to tertiary neurons of the vomeronasal cells.

All publications, patents and patent applications cited herein are incorporated herein by reference in their entirely.

Effects of the Invention

The present invention permits the selective visualization of functional neural pathways through a particular group of neurons, which could not have been achieved by tracing technique using a conventional trans-synaptic tracer protein. Further, the present invention does not have any of the problems observed in the tracing technique using a conventional trans-synaptic tracer protein, for example, serious immune responses caused by injection of the trans-synaptic tracer protein into an animal and individual differences due to injection technique.

Brief Description of the Drawings

Figure 1 shows the construction of WGA gene.

Figure 2 shows Western blotting of the WGA gene product (electrophoresis photograph).

Figure 3 shows WGA protein detection in N2a cells (microphotograph).

Figure 4 shows WGA mRNA detection and WGA protein detection in a section of the brain (microphotographs).

Figure 5 shows WGA protein detection in a section including the cerebellum (microphotograph).

Figure 6 shows WGA protein detection in the deep cerebellar nuclei (microphotographs).

Figure 7 shows WGA protein detection in various brain sections (microphotographs).

Figure 8 shows a schematic diagram of neural pathways originating from Purkinje cells.

Figure 9 shows WGA protein detection in a section including the vomeronasal organ (microphotographs).

Figure 10 shows axon detection and WGA protein detection in a section including axons of the vomeronasal organ (microphotographs).

Figure 11 shows WGA protein detection in a section of the brain

(microphotograph).

Figure 12 shows WGA protein detection in a section including the accessory olfactory bulb (microphotographs).

Figure 13 shows WGA protein detection in a section including the lateral olfactory tract (microphotographs).

Figure 14 shows WGA protein detection in a section including the medial amygdaloid nucleus, posteromedial cortical amygdaloid nucleus, and bed nucleus of stria terminalis (microphotographs).

Figure 15 shows a schematic diagram of neural pathways originating from vomeronasal sensory neurons.

CLAIMS

1. A transgenic animal into which a gene encoding a trans-synaptic tracer protein is introduced so as to direct specific expression in particular neurons.

2. The transgenic animal according to claim 1, wherein a promoter specific to the particular neurons is located upstream of the gene encoding the trans-synaptic tracer protein.

3. The transgenic animal according to claim 1 or 2, wherein the trans-synaptic tracer protein is wheat germ agglutinin.

4. A method for screening neuromimetic substances, which comprises:

administering a test substance to the transgenic animal according to any one of claims 1 to 3; and

selecting a neuromimetic substance from among the test substances by using as an indicator the trans-synaptic tracer protein expressed in neurons of the transgenic animal.

5. A neuromimetic substance obtainable by the screening method according to claim 4.

ABSTRACT

The present invention provides a transgenic animal into which a gene encoding a trans-synaptic tracer protein is introduced so as to direct specific expression in particular neurons. The use of this transgenic animal permits the selective visualization of functional neural pathways through a particular group of neurons, which could not have been achieved by tracing technique using a conventional trans-synaptic tracer protein.

Figure 1

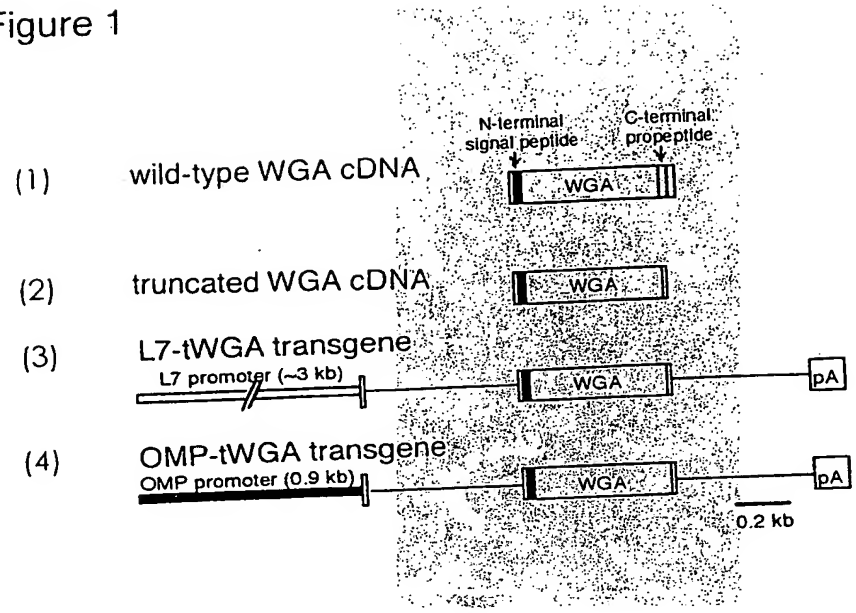


Figure 2

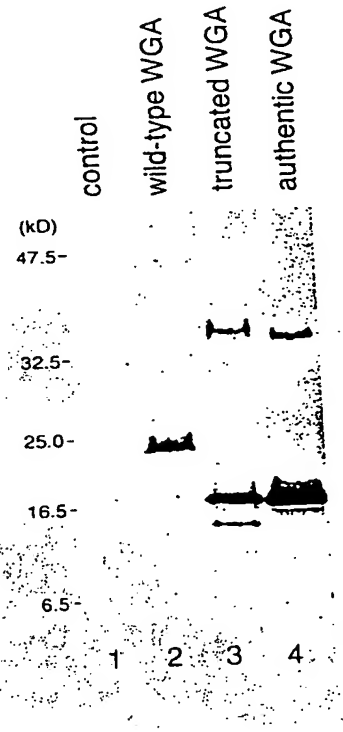
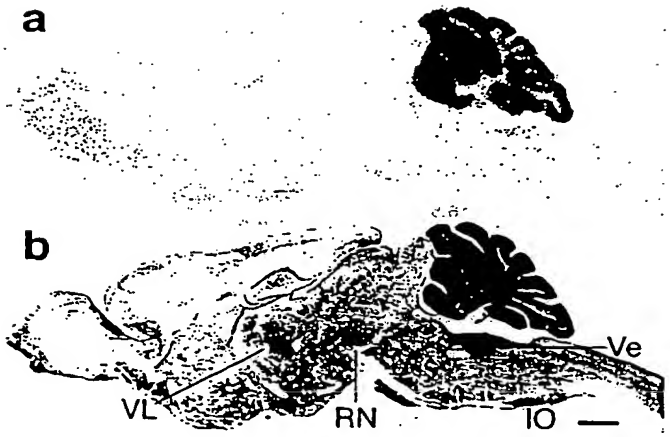


Figure 3

Figure 4

(1)

(2)



VL : thalamic ventrolateral nucleus

Ve : vestibular nucleus

RN : red nucleus

IO : inferior olivary nucleus

Figure 5

Figure 6

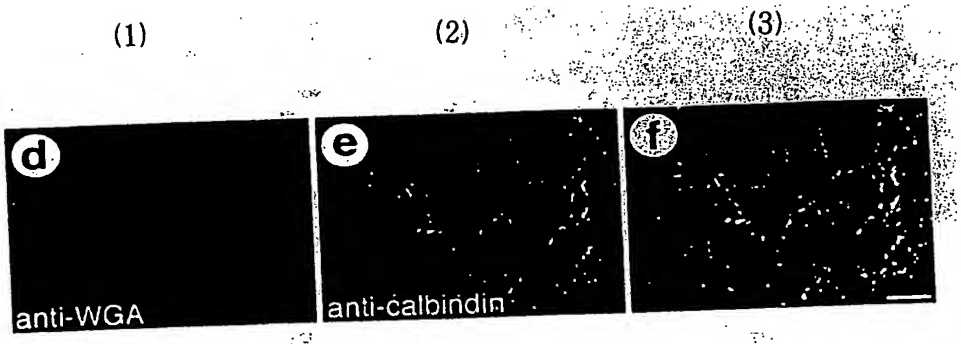
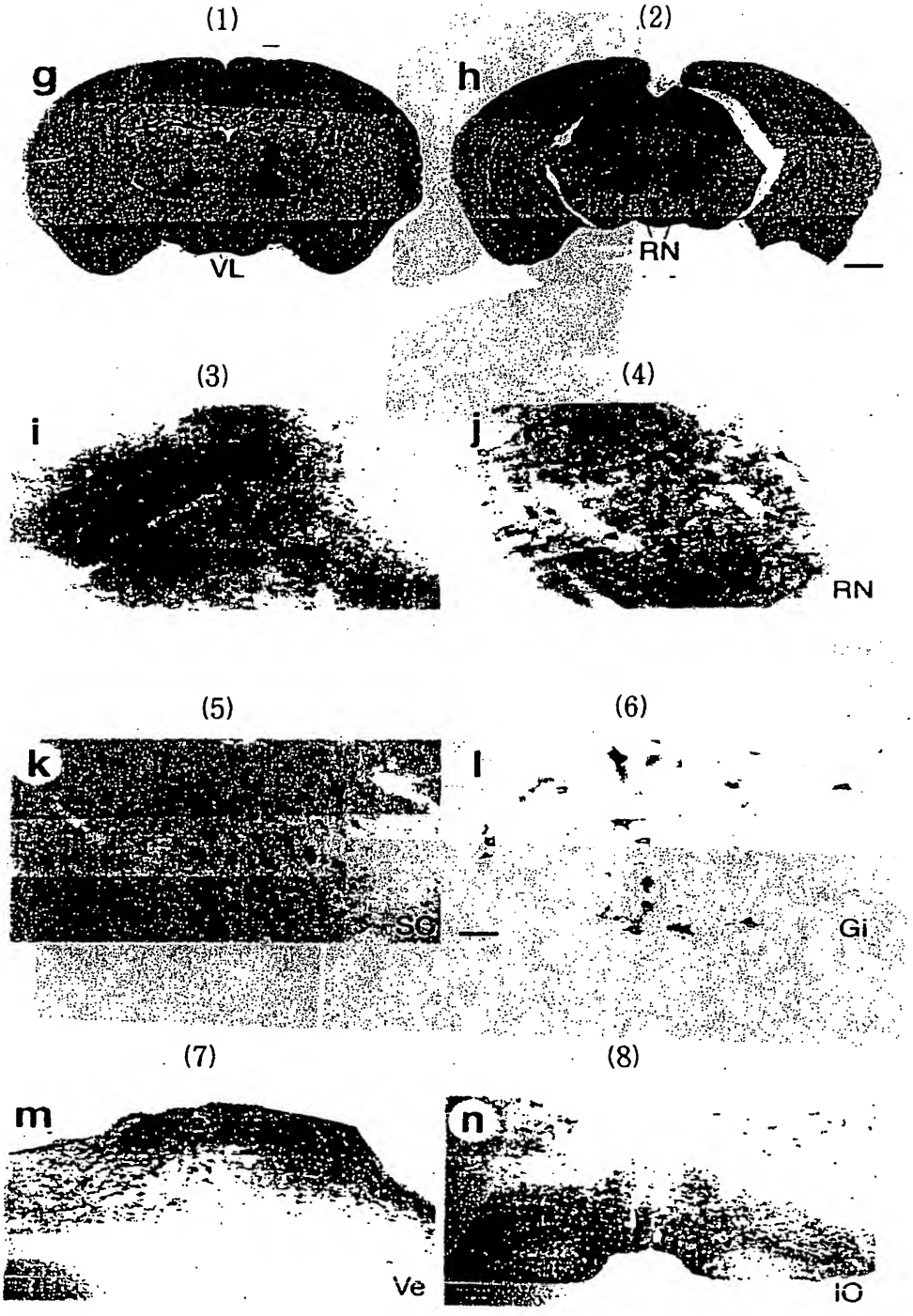


Figure 7



VL : thalamic ventrolateral nucleus

Ve : vestibular nucleus

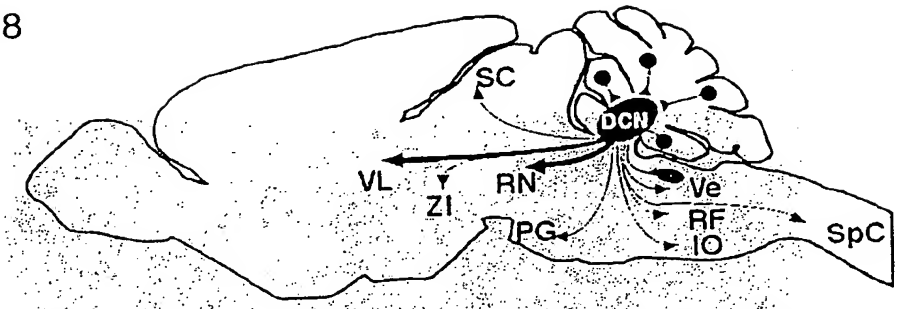
RN : red nucleus

SC : superior colliculus

Gi : gigant cellular reticular nucleus

IO : inferior olivary nucleus

Figure 8



DCN : deep cerebellar nuclei

SC : superior colliculus

VL : thalamic ventrolateral nucleus

ZI : zona incerta

RN : red nucleus

Ve : vestibular nucleus

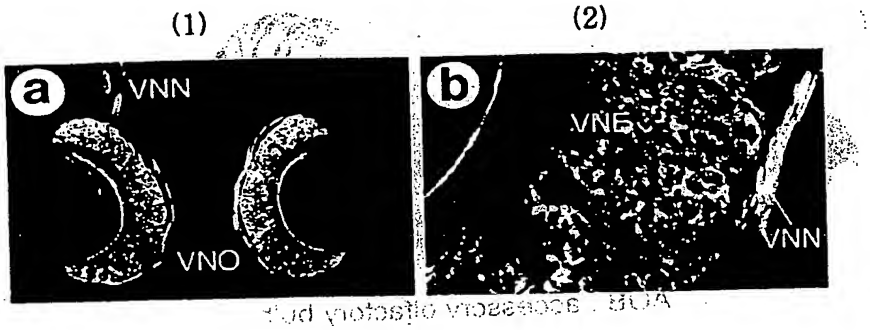
PG : pontine nuclei

RF : brain stem reticular formation

IO : inferior olivary nucleus

SpC : spinal cord

Figure 9

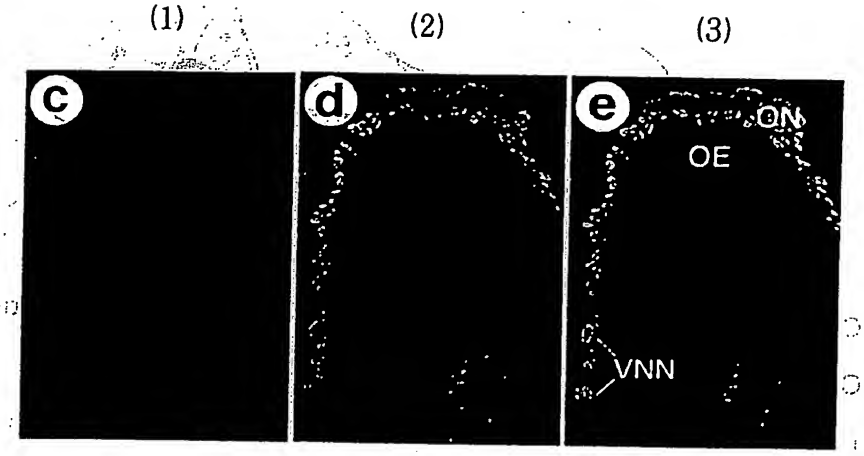


VNN : vomeronasal nerve bundle

VNO : vomeronasal organ

VNE : vomeronasal epithelium

Figure 10



ON : olfactory nerves

OE : olfactory epithelium

VNN : vomeronasal nerve bundle

Figure 11

AOB : accessory olfactory bulb

Figure 12

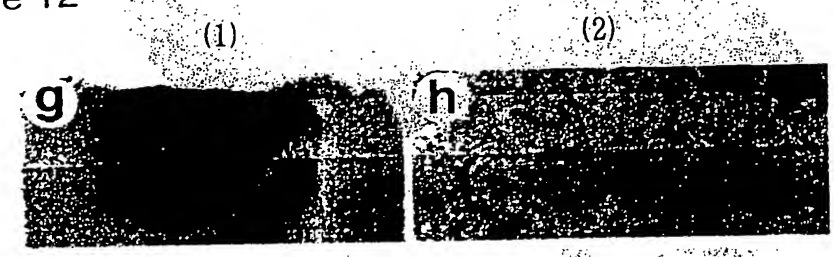


Figure 13

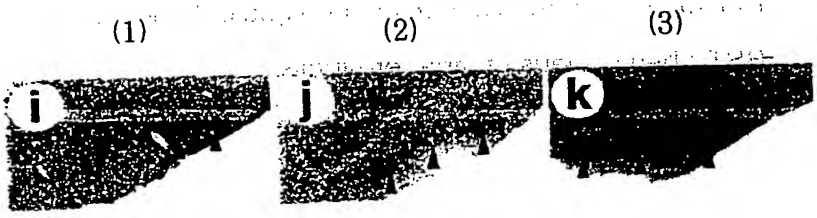
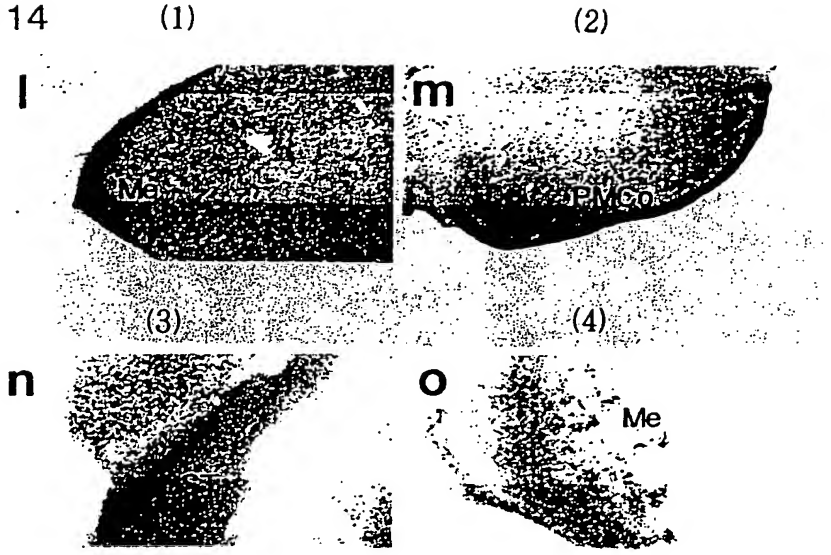


Figure 14

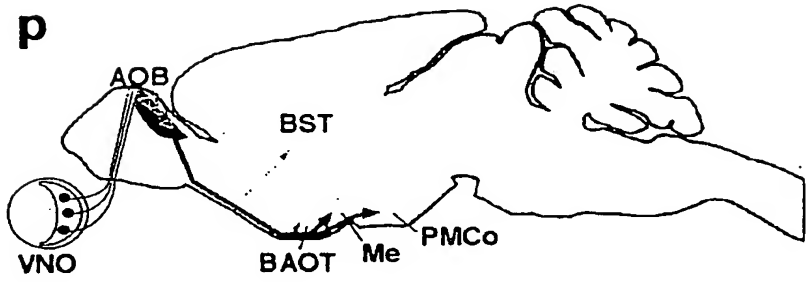


Me : medial amygdaloid nucleus

PMCo : posteromedial cortical amygdaloid nucleus

BST : bed nucleus of stria terminalis

Figure 15



AOB : accessory olfactory bulb

BST : bed nucleus of stria terminalis

VNO : vomeronasal organ

BAOT : bed nucleus of accessory olfactory pathway

Me : medial amygdaloid nucleus

PMCo : posteromedial cortical amygdaloid nucleus